Characterisation of the murine interferon-inducible T cell alpha chemoattractant (I-TAC).

By

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STATEMENT

Unless stated below, the author performed the work presented in this thesis. Dr Joanne Banyer provided the cultured dendritic cell line cDNA. Ann Prins prepared the histology slides. Anthony Murfett and Adam Wheatley carried out the construction of the recombinant baculovirus. Dr Surendran Mahalingam provided samples of cDNA used for the RSV and EAE studies and Michelle Solomon provided samples of cDNA used for the transplantation studies. All people mentioned here are located at the John Curtin School of Medical Research (JCSMR). The material herein has not been submitted in whole or in part for a degree at this, or any other university.

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Writing a PhD is similar to climbing a very high mountain. Mount Everest was thought to be impossible to climb but Sir Edmund Hillary concentrated on putting one foot in front of the other and eventually he got there. He of course remembered to stop putting one foot in front of the other when he got to the top, otherwise he would have fallen down the other side. This would've annoyed Sherpa Tenzing to no end as he was tied to him and Hillary was quite a bit heavier. Every high altitude mountain climb also requires a good support crew. For my PhD Mountain, I'm very indebted to my guide and sherpa, Dr Scott Thomson. Professor Ian Ramshaw, and Drs. Joanne Banyer, Suresh Mahalingam, Klaus Matthaei and Maryanne Shoobridge have all provided invaluable resources and advice from base camp throughout this expedition.

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PUBLICATIONS

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ABSTRACT

A large sub-family of cytokine proteins, termed chemokines, are important mediators of immune cell communication and primarily function to direct the migration of cells during immune responses. The murine homologue of the human chemokine interferon-inducible T cell alpha chemoattractant (I-TAC) was isolated by a cDNA subtraction technique from a dendritic cell line differentiated with IFN-γ and TNF-α. Murine I-TAC is 67% homologous to HuI-TAC and ~35% homologous to the murine chemokines Crg-2 and MuMig respectively. There are four cysteine residues with the first two being separated by one amino acid, and as a result is a member of the non-ELR CXC chemokine family. The open reading frame is 100 amino acids long with a predicted 21 amino acid leader sequence. The predicted leader sequence coincides with the leader sequence for HuI-TAC and suggests murine I-TAC is a 79 amino acid residue mature protein. By using a baculovirus protein expression system, it was demonstrated that the isolated murine I-TAC cDNA is functional and attracts activated splenocytes.

Using semi-quantitative RT-PCR analysis of two cytokine-stimulated murine dendritic cell lines, MTHC-D2 and JAWS II, as well as Con A activated splenocytes from a panel of knockout mice, it was elucidated that IFN-γ and anti-CD40 Ab separately, and in synergy, enhance murine I-TAC expression. IFN-γ-regulated I-TAC expression is primarily mediated through IRF-1 activation and IFN-α/β pathway down regulates murine I-TAC mRNA levels. Expression of I-TAC was reduced in the DC lines when exposed to the cytokine, IL-4. This suggests that I-TAC is primarily involved in cell mediated immune responses.

Since I-TAC is secreted from IFN-γ stimulated DCs the level of murine I-TAC mRNA expression was assessed by RT-PCR in different disease models. Expression levels of I-TAC increased in correlation with respiratory syncytial viremia and influenza viremia during infection. The expression of I-TAC after IFN-γ stimulation and expression during viral infection further verifies that I-TAC has a function in cell mediated immune responses, probably by recruiting $CXCR3^+$ T cells. In contrast however, expression of I-TAC was not detected by RT-PCR during a vaccinia virus infection, whereas previously, expression of the homologous chemokines Mig and Crg-2 have been

detected (Mahalingam *et al.*, 2000; Mahalingam and Karupiah, 2000). This suggests that vaccinia virus may have a mechanism to inhibit I-TAC expression.

The expression of I-TAC was also assessed in the CNS of wild type and IFN-γ $R^{-/-}$ mice during the myelin oligodendrocyte (MOG_{35-55}) peptide autoimmune disease model, experimental autoimmune encephalomyelitis (EAE). Peak I-TAC expression was detected in wild type mice on day 14 when the mice begin to recover, whereas no increase in I-TAC expression was detected in IFN-γ $R^{-/-}$ mice which develop severe EAE and die. These expression studies do not provide an understanding to the functional role of I-TAC during EAE, however, previously it has been shown that antibodies to a functional domain of CXCR3 protect mice from EAE (Arimilli *et al.*, 2000). It is therefore likely that murine I-TAC may act to enhance neuroinflammatory processes and exacerbate EAE disease.

The last disease models investigated were two forms of rejected and non-rejected transplantation grafts. Murine I-TAC was expressed early during both rejected and non-rejected allograft transplants, indicating that I-TAC may be expressed as a result of general damage following transplantation surgery rather than as a direct result of specific allograft rejection. The study analyzing tissues from rejected xenograft transplantation showed varying results throughout the time course, which may indicate differing levels of inflammation and cellular recruitment in different mice. Although the result does not appear to show a clear trend of I-TAC expression in the xenograft transplantation model it does show that cells expressing I-TAC migrate into the graft. These cells may be DCs or other APCs, which then express I-TAC in order to recruit T cells.

To investigate the biological role of I-TAC *in vivo* a recombinant vaccinia virus encoding this chemokine (rVV I-TAC) was constructed. The recombinant virus was attenuated *in vivo* compared to the control virus (VV-WR). The recruitment of mononuclear cells into the site of infection was higher and occurred earlier in mice challenged with rVV I-TAC than those infected with the control virus. Subsequently, both C57BL/6 and athymic Swiss nude mice inoculated with rVV I-TAC were able to resolve infection significantly more successfully than those infected with VV-WR.

The characteristics of murine I-TAC suggest it is an important mediator of immune cell communication. The research presented here has extended the knowledge about this potent chemokine and may subsequently augment the development of vaccines and therapeutics containing chemokines such as I-TAC to improve their effectiveness.

TABLE OF CONTENTS

ABBREVIATIONS

ABE	animal breeding establishment
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
Crg-2	cytokine responsive gene
CMI	cell mediated immunity
CTL	cytotoxic T lymphocyte
DTH	Delayed-type hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
FCS	foetal calf serum
GAS	gamma activation site
GM-CSF	granulocyte-macrophage colony-stimulating factor
HA	haemagglutinin
HAU	haemagglutinin units
HI	humoral immunity
HIV	human immuno deficiency virus
i.n	intranasal
i.p	intraperitoneal
i.v	intravenous
IFN	interferon
IL	interleukin
IP-10	interferon-inducible protein-10
IRF	interferon regulatory factor
ISGF	interferon-α-stimulated gene response factor
I-TAC	interferon-inducible T cell alpha chemoattractant
JAK	Janus kinase
mAbs	monoclonal antibodies
MCP-1	monocyte chemotactic protein-1
Mig	monokine induced by IFN-γ
MIP-1α/β	macrophage inflammatory protein-1α/β
MOG	myelin oligodendrocyte glycoprotein

MOI	multiplicity of infection
mRNA	messenger RNA
NK	natural killer
PF4	platelet factor 4
PFU	plaque forming unit
RANTES	regulated on activation, normal T cell expressed and secreted
r	recombinant
rpm	rotations per minute
RT-PCR	reverse-transcriptase polymerase chain reaction
STAT	signal transducers and activators of transcription
Th	T helper
TNF	tumour necrosis factor
VV	vaccinia virus
VV-WR	Western reserve strain of vaccinia virus

CHAPTER 1.

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW.

1.1 General overview

There are many cells of the immune system, each carrying out different functions to overcome infection and keep the host in a healthy state. Host defense mechanisms are numerous and range from relatively primitive, constitutively expressed, non-specific defenses, to sophisticated adaptive mechanisms that are specifically induced in response to foreign antigen (invading pathogens). The process of immune cell activation requires recognition of invariant molecular structures common to a variety of pathogens by innate immune cells; these are called pathogen-associated molecular patterns (PAMPs). Well known examples of PAMPs include lipopolysaccharides and teichoic acids, both shared by a variety of bacteria, and unmethylated CpG motifs found in microbial but not mammalian DNA. Host organisms have evolved cellular receptors with broad specificities that can recognize PAMPs from infectious microbes (Kopp and Medzhitov, 1999; Muzio and Mantovani, 2000). The result of PAMP recognition is the activation of the innate, non-specific immune cells such as natural killer cells (NKs), mast cells, macrophages, eosinophils, neutrophils and dendritic cells (DCs). Although the role of innate immunity was originally considered to primarily defend the host early in infection, it is now clear that there is an important reciprocal relationship between innate and adaptive immune responses throughout an infection. Antigen presenting cells (APCs), primarily DCs, as well as cell to cell communication mediators, including cytokine and chemokine proteins, have an essential role in orchestrating this process as well as determining the type of effector response that is ultimately generated towards a pathogen. These cytokines and chemokines are produced at sites of infection following the pathogens interaction with innate immune cells or from damaged tissues. The production of cytokines and chemokines not only further activates the innate immune cell population but also drives the recruitment and activation of adaptive immune cells to eliminate the invading pathogen (reviewed in Banyer *et al.*, 2000). The adaptive immune response consists of pathogen-specific T- and B- lymphocyte responses that are stimulated through their interactions with APCs like macrophages and DCs.

The nature of the immune response that develops is determined by the nature of the invading pathogen and its affect on the infected tissue and innate immune cells. This

interaction determines the type of cytokines, chemokines and other mediators that are released into the microenvironment situated around the site of infection. In turn, these released factors stimulate innate immune cells, including immature DCs, to develop specific immunoregulatory properties that reflect the identity and state of infection, and subsequently direct the adaptive immune response towards the invading pathogen (reviewed in Baggiolini *et al.*, 1997; Rollins, 1997). Thus, both cytokines and chemokines are crucial elements in the progression and regulation of the immune response.

1.2 Cytokines – an overview

Cytokines are small protein messengers that regulate the development and expression of a wide range of immune responses. A subfamily of cytokines, termed chemokines, specifically control trafficking of immune cells. As such, both cytokines and chemokines are critical in the regulation of cells that participate in both innate and adaptive immune responses. Cytokines and chemokines may act both in highly localized environments but also in a systemic manner, and they may, themselves, directly mediate anti-pathogen effects (reviewed in Banyer *et al.*, 2000).

Cytokines may be grouped into two broad categories, firstly, those that act to stimulate T helper cell (Th) type one (Th1), cell mediated immune (CMI) responses, which mainly protect against intracellular viral, bacterial, fungal and protozoan pathogens, (e.g. interleukin-12, IL-12, and interferon gamma, IFN-γ, cytokines). The second group of cytokines stimulate type two (Th2), humoral immune (HI) responses involved in protection against extra cellular infections such as parasites and extracellular bacteria (including interleukins 4, 5, 6, 10 and 13).

The cytokine microenvironment initiated at the site of infection also influences innate cell populations, including macrophages, natural killer cells (NKs), and DCs, which subsequently influence the CMI or HI adaptive immune responses. This cytokine microenvironment is therefore a key determinant for developmental pathway of the precursor cells and the overall immune response (O'Garra, 1998). For example, viral pathogens usually stimulate innate immune cells to produce factors that promote development of Th1 cells. Thus, DC and macrophages commonly secrete large

amounts of IL-12, and subsequently NK cells produce IFN-γ, the effect of which is to drive Th0 cells to differentiate into Th1 cells. Conversely, the early production of IL-4 for example, by a variety of innate cell types in response to extracellular pathogens such as helminths, directs Th2 cell development (Seder and Paul, 1994; Bendelac *et al.*, 1997).

To ensure inflammatory cytokine-mediated immune responses do not unintentionally damage the host, the immune system has protective mechanisms. An example of this is the reciprocal cross-regulatory effects of Th1 and Th2 effector cytokines, such as IFN-γ and IL-4 respectively. In addition to this, Th1 and Th2 cytokines can have down regulatory effects on cells of the innate immune system. As a result the innate and adaptive immune responses are intimately linked and controlled by sets of cytokines and their respective receptors which act to generate the most effective form of immunity suitable for protection against each given pathogen (Banyer *et al.*, 2000).

1.2.1 IFN-γ

The interferon (IFN) cytokines were initially associated with antiviral activity and are a key element in CMI (reviewed in Stark *et al.*, 1998). They are also associated with a multitude of other functions including the inhibition of cell growth and proliferation and regulation of cell apoptosis, and numerous effects on the immune system. In addition, IFNs regulate protein expression and processing necessary for encoding the major histocompatibility complex (MHC), induction of $CD4^+$ T helper cell phenotype development, macrophage activation, and also chemokine and cytokine induction and inhibition. The IFNs are subdivided into two main families, type I IFNs (also known as IFN-α/β) and type II IFN (IFN-γ). The type I IFNs have four subfamilies, these being IFN-α, IFN-β, IFN-ω, and IFN-τ whereas there is only IFN-γ in the type II family (Vilcek and Oliveira, 1994; Stark *et al.*, 1998; Platanias and Fish, 1999). Although both classes of IFNs exhibit antiviral activity, the IFN-α/β class are more widely expressed and play an important role in the innate immune response. IFN-γ, however, tends to have an important role in the activity of the NK cell, innate immune responses and the Th1 type adaptive immune response (Schindler, 1999).

1.2.2 IL-12

The predominant role of IL-12 as a determinant of Th1 cell development is now well established (reviewed in Hsieh *et al.*, 1993; Trinchieri, 1995). IL-12 signals primarily through the transcription factors, signal transducer and activator of transcription-3 (STAT3) and STAT4 (Jacobson *et al.*, 1995; Szabo *et al.*, 1995), although the recent discovery of T-bet provides clear evidence for further important lineage-specific transcription factors in Th1 cell development (Szabo *et al.*, 2000). T-bet, which belongs to the T-box family of transcription factors, is rapidly and selectively induced in developing Th1 cells (but not Th2 cells). This in turn, upregulates the expression of IFN-γ. This factor is inducible by IL-12, a process enhanced by IL-18 and diverts naive T cells into the Th1 differentiation pathway. T-bet may even convert committed Th2 cells into Th1 cells, at least partly via repression of IL-4 and IL-5 gene expression (Szabo *et al.*, 2000).

Functional receptors for IL-12 are expressed on recently activated and committed naive T cells and Th1 cells, but are lost during Th2 cell differentiation. The unresponsiveness of Th2 cells to IL-12 may thus be explained by down regulation of the IL-12 receptor β2 chain (IL-12Rβ2) by IL-4. Conversely, IFN-γ acts to upregulate IL-12Rβ2 expression by Th1 cells and counteracts the activity of IL-4 in this respect (Rogge *et al.*, 1997; Szabo *et al.*, 1997). This shows that once the immune response pathway has been determined, T cells subsequently act to amplify the cytokine signals that enhance the required immune cells while also down regulating the other, unnecessary arm of the T cell immune response.

1.2.3 IL-4

Just as IL-12 is a key factor in Th1 cell lineage commitment, IL-4 appears to play a similar role in initiating Th2 cell development (reviewed in Nelms *et al.*, 1999). Indeed, the influence of IL-4 on naive Th cells appears to be dominant over that of IL-12, such that threshold levels of IL-4 at the point of induction of an immune response will lead to Th2 cell development and up-regulated IL-4 production (Le Gros *et al.*, 1990; Seder and Paul, 1994). IL-4 signals through the transcription factor STAT6 to upregulate its own expression. However, STAT activation is a transient event and is unlikely, by itself, to determine Th cell phenotype development as this is a process which takes several days

(Abbas *et al.*, 1996; Lederer *et al.*, 1996; O'Garra, 1998). Additional lineage-specific transcription factors also appear to be required; c-Maf increases IL-4 expression (Ho *et al.*, 1996), whilst GATA-3 induces expression of several Th2 factors in both developing and committed T cells and may inhibit IFN-γ secretion (Zhang *et al.*, 1997; Zheng and Flavell, 1997). The source of IL-4 early in the immune response, which is important for Th2 cell differentiation, remains controversial. Non-T cells, including DCs, mast cells, basophils and eosinophils are prime candidates, as are CD4+ and CD4-CD8- T cells of the NK1+ subset and LACK (*Leishmania* homologue of receptors for activated kinase C)-specific CD4+ T cells expressing Vβ4 and Vβ8 TCR (Seder and Paul, 1994; Julia *et al.*, 1996; Bendelac *et al.*, 1997). Recently, it has been found that certain pathogens trigger IL-4 secretion from DCs themselves (d'Ostiani *et al.*, 2000). In addition to this, a key role for NK1+ cells in Th2 cell development was suggested by their capacity to produce large amounts of IL-4 immediately following activation (Bendelac *et al.*, 1997). However, mice deficient in CD1, the selecting antigen for NK1+ cells, were able to mount Th2-type responses in the absence of this cell subset (Smiley *et al.*, 1997). Whilst IL-4 is a clearly a critical mediator of Th2 differentiation, the mechanisms underlying its activity are yet to be fully understood.

1.2.4 Tumour necrosis factor (TNF)

There are four major TNF family members, TNF-α, LT-α, LT-β and LIGHT, which interact with four receptors termed p55 TNF receptor, p75 TNF receptor, the lymphotoxin β receptor (LT-βR) and the herpes virus entry mediator (HVEM) (reviewed in Wallach *et al.*, 1999). The members of the TNF family have a wide range of functions during both innate and adaptive immune responses including regulating of inflammatory responses and induction of apoptosis (reviewed in Wallach *et al.*, 1999). Dramatic increases of TNF-α have been observed after brain injury, such as a stroke or head trauma (Bruce *et al.*, 1996). By using TNF-α receptor knockout mice, it was demonstrated that TNF protects neurons by stimulating antioxidant pathways, especially the antioxidant enzyme superoxide dismutase (Bruce *et al.*, 1996). In contrast TNF-α has been demonstrated to be critical in inducing neuronal apoptosis during endotoxic shock (de Bock *et al.*, 1998). In this latter study the presence of oxygen radicals, normally inhibited by antioxidant factors, were also necessary for cell death. These

studies indicate that the interaction between TNF and antioxidant pathway activity is an essential determinant of neuronal apoptosis.

There are many more cytokines expressed throughout the body (e.g. IL-1 through to IL-18, TGF-α, TGF-β and GM-CSF) and while the role of many of these have been well characterised, the exact roles of the more recently identified cytokines are still being established. Many can act in concert with each other or function antagonistically. The importance of each cytokine may vary depending on specific diseases, for example IL-5 appears to be important during asthma and lung allergies.

1.3 Chemokines; A subfamily of cytokines

Cells of the immune system need to identify and migrate to sites of infection. A large sub-family of cytokine proteins, termed chemokines, function to direct the migration of cells during immune responses. This occurs by the release of chemokines from infected cells and activated bystander cells, which disperse and interact with receptors expressed on immune cells in the vicinity. The first chemokine to be identified was during the mid-1960s and is now termed platelet factor 4 (PF4). However, it was not until studies examining inflammation and cell migration that the second chemokine, interleukin-8 (IL-8), was cloned and the chemokine family established (Schmid and Weissmann, 1987; Walz *et al.*, 1987; Gale and McColl, 1999). Research into chemokines, moreover, still did not develop momentum for another eight years. In 1995 Cocchi *et al.*, demonstrated that three chemokines, known as RANTES, MIP-1α, and MIP-1β, could inhibit the human immuno-deficiency virus (HIV) which causes the Acquired Immune Deficiency Syndrome, or AIDS (Cocchi *et al.*, 1995). Shortly after, it was demonstrated that HIV required the presence of a chemokine receptor to gain entry into host cells to initiate infection which significantly increased chemokine research (Alkhatib *et al.*, 1996; Bates, 1996; Bleul *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996; Oberlin *et al.*, 1996). Since then, a large number of chemokines have been identified and characterised which function throughout the body. Approximately 55 distinct human chemokines have been identified to date. Mouse and rat homologues have also been identified for many of these, enabling the function of chemokines to be examined in convenient animal models.

1.4 Chemokine classification

Most chemokines are small proteins being only 92 to 125 amino acids in length (8-12 kDa) with leader sequences of 20 to 25 amino acids directing the chemokine to be secreted extracellularly. The chemokine family is generally divided into four subfamilies based on the presence and location of the first conserved cysteine residues and are termed CXC (α), CC (β), C (γ), and CX_3C (δ) chemokines, with the X denominating any other amino acid (Fig 1.1). Due to the rapidly growing number of chemokines being identified, research groups have often reported the same protein under different names. Recently a new chemokine classification has been suggested for consideration as an internationally approved nomenclature (Zlotnik and Yoshie, 2000). A summary of the known human chemokines and mouse homologues, with their new and old nomenclature, as well as the receptor for which they bind, is shown in table 1.1.

1.4.1 CXC chemokines

The CXC chemokines can be further subdivided based on the presence or absence of an N-terminal region sequence ELR (glutamine-leucine-arginine amino acids), which lies between the N-terminus and the first cysteine residue and is necessary for binding to IL-8 receptors on the surface of neutrophils (Fig 1.1). Hence those chemokines which contain ELR sequences function as neutrophil chemoattractants (reviewed in Mackay, 1997; Rollins, 1997; Rossi and Zlotnik, 2000). In humans, the genes of nearly all CXC chemokines are clustered on chromosome 4 (predominantly 4q12-q13) (reviewed in Baggiolini *et al.*, 1997; Rollins, 1997).

Chemokine family

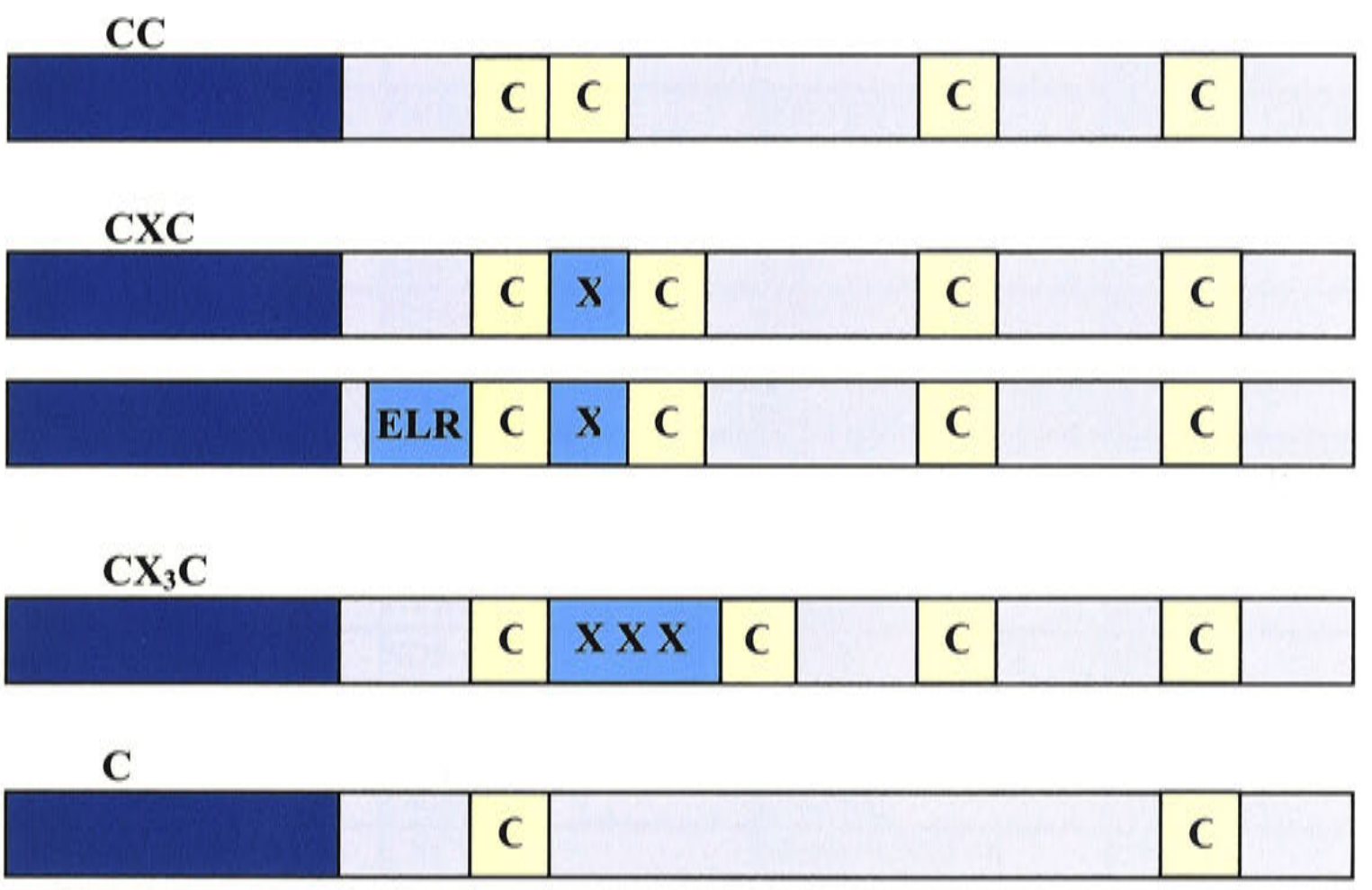


Figure 1.1: Schematic diagram showing the relationship between the four chemokine subfamilies. The dark blue regions indicate the leader sequence and the conserved cysteine residues are also shown. Note that the CXC chemokines are further divided into ELR and non-ELR subgroups.

Table 1.1: Known human chemokines with their new and old nomenclature, homologous mouse ligand, and receptor to which they bind. Adapted from Zlotnik and Yoshie, 2000.

CXC chemokine family

Systematic Name	Human Chromosome	Human Ligand	Mouse Ligand	Chemokine Receptor(s)
CXCL1	4q12-q13	GRO-α/MGSA-α	GRO/KC?	CXCR2 > CXCR1
CXCL2	4q12-q13	GRO-β/MGSA-β	GRO/KC?	CXCR2
CXCL3	4q12-q13	GRO-γ/MGSA-γ	GRO/KC?	CXCR2
CXCL4	4q12-q13	PF4	PF4	Unknown
CXCL5	4q12-q13	ENA-78	LIX?	CXCR2
CXCL6	4q12-q13	GCP-2	CK-3	CXCR1, CXCR2
CXCL7	4q12-q13	NAP-2	Unknown	CXCR2
CXCL8	4q12-q13	IL-8	Unknown	CXCR1, CXCR2
CXCL9	4q21.21	Mig	Mig	CXCR3
CXCL10	4q21.21	IP-10	Crg-2	CXCR3
CXCL11	4q21.21	I-TAC	Unknown	CXCR3
CXCL12	10q11.1	SDF-1α/β	SDF-1	CXCR4
CXCL13	4q21	BLC/BCA-1	BLC/BCA-1	CXCR5
CXCL14	Unknown	BRAK	BRAK	Unknown
(CXCL15)	Unknown	Unknown	Lungkine	Unknown
CXCL16	Unknown	CXCL16	Murine CXCL16	CXCR6 *

C chemokine family

Systematic Name	Human Chromosome	Human Ligand	Mouse Ligand	Chemokine Receptor(s)
XCL1	1q23	Lymphotactin/SCM-1α ATAC	Lymphotactin	XCR1
XCL2	1q23	SCM-1β	Unknown	XCR1

CX_3C chemokine family

Systematic Name	Human Chromosome	Human Ligand	Mouse Ligand	Chemokine Receptor(s)
CX3CL1	16q13	Fractalkine	Neurotactin	CX3CR1

* A recently identified chemokine (Matloubian *et al.*, 2000).

Matloubian, M., David, A., Engel, S., Ryan, J.E. and Cyster, J.G. (2000). "A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo." *Nat Immunol* 1(4): 298-304.

Table 1.1 continued: Known human chemokines with their new and old nomenclature, homologous mouse ligand and receptor to which they bind. Adapted from Zlotnik and Yoshie, 2000.

CC chemokine family

Systematic Name	Human Chromosome	Human Ligand	Mouse Ligand	Chemokine Receptor(s)
CCL1	17q11.2	I-309	TCA-3, P500	CCR8
CCL2	17q11.2	MCP-1/MCAF	JE?	CCR2
CCL3	17q11.2	MIP-1α/LD78	MIP-1α	CCR1, CCR5
CCL4	17q11.2	MIP-1β	MIP-1β	CCR5
CCL5	17q11.2	RANTES	RANTES	CCR1, CCR3, CCR5
(CCL6)		Unknown	C10, MRP-1	Unknown
CCL7	17q11.2	MCP-3	MARC?	CCR1, CCR2, CCR3
CCL8	17q11.2	MCP-2	MCP-2?	CCR3
(CCL9/10)		Unknown	MRP-2, CCF18 MIP-1	Unknown
CCL11	17q11.2	Eotaxin	Eotaxin	CCR3
(CCL12)		Unknown	MCP-5	CCR2
CCL13	17q11.2	MCP-4	Unknown	CCR2, CCR3
CCL14	17q11.2	HCC-1	Unknown	CCR1
CCL15	17q11.2	HCC-2/Lkn-1/MIP-1	Unknown	CCR1, CCR3
CCL16	17q11.2	HCC-4/LEC	LCC-1	CCR1
CCL17	16q13	TARC	TARC	CCR4
CCL18	17q11.2	DC-CK1/PARC AMAC-1	Unknown	Unknown
CCL19	9p13	MIP-3β/ELC/exodus-3	MIP-3β/ELC/exodus-3	CCR7
CCL20	2q33-q37	MIP-3α/LARC/exodus-1	MIP-3α/LARC/exodus-1	CCR6
CCL21	9p13	6Ckine/SLC/exodus-2	6Ckine/SLC/exodus-2/TCA-4	CCR7
CCL22	16q13	MDC/STCP-1	ABCD-1	CCR4
CCL23	17q11.2	MPIF-1	Unknown	CCR1
CCL24	7q11.23	MPIF-2/Eotaxin-2	Unknown	CCR3
CCL25	19p13.2	TECK	TECK	CCR9
CCL26	7q11.23	Eotaxin-3	Unknown	CCR3
CCL27	9p13	CTACK/ILC	ALP/CTACK/ILC	CCR10
CCL28	unknown	CCL28	Mouse CCL28	CCR10 *

* A recently identified chemokine (Wang *et al.*, 2000).

Wang, W., Soto, H., Oldham, E.R., Buchanan, M.E., Homey, B., Catron, D., Jenkins, N., Copeland, N.G., Gilbert, D.J., Nguyen, N., Abrams, J., Kershenovich, D., Smith, K., McClanahan, T., Vicari, A.P. and Zlotnik, A. (2000). "Identification of a novel chemokine (CCL28), which binds CCR10 (GPR2)." *J Biol Chem* 275(29): 22313-22323.

1.4.1.1 GRO-α/β/γ (CXCL1/2/3)

The three members of the GRO family (GRO α, β and γ) are closely related chemokines, sharing greater than 86%+ amino acid identity and all contain the ELR motif required for neutrophil recruitment (Haskill *et al.*, 1990). All three GROs bind to the CXCR2 receptor, expressed on neutrophils (White *et al.*, 1998). GRO-α, the first to be identified was isolated from transformed hamster cells that had lost the ability for growth control (Anisowicz *et al.*, 1987). It has however, also been referred to as melanoma growth stimulatory activity, or MGSA, as it has mitogenic effects on human melanoma cells (Richmond *et al.*, 1988). The expression of all three GROs appears to be regulated by two pathways, a cytokine (TNF-α/IL-1) induced signal and also a growth related pathway (Anisowicz *et al.*, 1991).

1.4.1.2 SDF-1 (CXCL12)

The non-ELR CXC chemokine, stromal cell-derived factor-1 (SDF-1) can activate and recruit monocytes, neutrophils, and peripheral blood lymphocytes and binds onto the receptor CXCR4. SDF-1 is a powerful HIV suppressive factor which probably occurs due to competition with the virus for the binding to the cell surface receptor (Bleul *et al.*, 1996; Oberlin *et al.*, 1996; Baggiolini *et al.*, 1997). SDF-1 occurs in two alternative splicing variants (SDF-1α and SDF-1β). One of the few exceptions to the clustering of CXC chemokines on chromosome 4 is SDF-1, which is encoded on chromosome 10 (10q11.1) (Shirozu *et al.*, 1995). SDF-1α stimulates the proliferation of B cell progenitors and was also termed pre-B cell growth stimulating factor (PBSF). Human SDF-1α is the more abundant splicing variant and the mature protein consists of 68 amino acids (Bleul *et al.*, 1996; Oberlin *et al.*, 1996; Baggiolini *et al.*, 1997).

1.4.1.3 Mig (CXCL9)

Monokine induced by IFN-γ (Mig), like SDF-1 is a non-ELR CXC chemokine. It is induced from monocytes and macrophages, specifically in response to IFNγ (Rollins, 1997). In addition to this, Mig has been observed in autoimmune lesions and some viral infections indicating that Mig is important during Th1 responses (Baggiolini *et al.*,

1997). Mig binds to the receptor CXCR3 which is found on NK cells, Th1 cells and not surprisingly acts as a potent chemoattractant for these cells (Liao *et al.*, 1995).

1.4.1.4 IP-10 (CXCL10)

The non-ELR CXC chemokine, Interferon-inducible protein-10 (IP-10, or Crg-2 in the mouse) is highly homologous to Mig and therefore carries many similarities in function including binding to the same receptor (CXCR3) and acting as a chemoattractant for activated $CD4^+$ T lymphocytes, monocytes, NK cells and also B lymphocytes (Taub *et al.*, 1993; Farber, 1997). The genes for human Mig and IP-10 are adjacent with a head-to-tail orientation with their start codons only 16 kb apart (Lee and Farber, 1996). Being located so closely and having similar homology and function, it is possible that the genes for Mig and IP-10 arose from gene duplication. One might therefore expect that there is a redundancy in expression or function but this is not the case, since they have different expression patterns during infections with either *Toxoplasma gondii* or vaccinia virus (VV). MuMig was expressed at the highest level in the liver in both disease models whereas Crg-2 co-localized with the respective pathogen and was expressed at high levels in various organs throughout the animal (Amichay *et al.*, 1996).

1.4.1.5 Human I-TAC (CXCL11)

Recently a new chemokine, interferon-inducible T cell alpha chemoattractant (I-TAC), has been isolated from a cDNA library derived from human peripheral blood mononuclear cells (Cole *et al.*, 1998). This chemokine has also been isolated by others and referred to as H174, β-R1, SCYB11, IP-9 and CXCL 11 (Rani *et al.*, 1996; Jacobs *et al.*, 1997; Laich *et al.*, 1999; Tensen *et al.*, 1999a). Expression of human I-TAC has been detected in cultured primary monocytes, cultured fetal astrocytes, a microglial cell line, astrocytoma cells, atheroma-associated cells, bronchial epithelial cells, polymorphonuclear neutrophils and keratinocytes (Rani *et al.*, 1996; Cole *et al.*, 1998; Gasperini *et al.*, 1999; Mach *et al.*, 1999; Sauty *et al.*, 1999; Tensen *et al.*, 1999b). Moderate expression has also been detected in human CNS, pancreas, lung, thymus and spleen (Cole *et al.*, 1998; Luo *et al.*, 1998). The mature I-TAC protein is a non-ELR CXC chemokine and consists of 94 amino acids. *In vitro* studies have demonstrated that expression of human I-TAC appears to be up-regulated following exposure to mainly

IFN-α, IFN-β and IFN-γ plus other cell regulators such as IL-1, LPS and TNF-α (Rani *et al.*, 1996; Cole *et al.*, 1998). Human I-TAC is homologous to human Mig and IP-10 and also uses the CXCR3 receptor but is more potent than these two chemokines. (Rani *et al.*, 1996; Jacobs *et al.*, 1997; Cole *et al.*, 1998). The variation in potency by these three chemokines may induce a chemical gradient which effector cells could migrate down to the site of infection (Cole *et al.*, 1998). Human I-TAC is a potent chemoattractant for activated T lymphocytes, NK cells and monocyte-like cells (Cole *et al.*, 1998; Luo *et al.*, 1998; Gasperini *et al.*, 1999). Very recently it was demonstrated that human I-TAC can specifically attract T cell receptor (TCR) $\alpha\beta^+CD8^+$ single positive T cells, TCR $\gamma\delta^+$ T cells, and NK-type cells in human thymus (Romagnani *et al.*, 2001b). One particular focus of I-TAC research is in diseases of the nervous system. Stimulation of astrocytes with IFN-γ and IL-1 results in a 400,000 fold increase in human I-TAC mRNA expression (Cole *et al.*, 1998). It has also been suggested that human I-TAC could be involved in the pathophysiology of neuro-inflammatory disorders such as multiple sclerosis and in the migration of Th1 cells during IFN-dominated immune responses (Cole *et al.*, 1998).

1.4.2 CC chemokines

The largest group of chemokines is the CC family of which there are 27 known human members. The genes of the human CC family are generally all located on chromosome 17 (predominantly 17q11.2-12) (Baggiolini *et al.*, 1997; Rollins, 1997). Two well characterised CC chemokines are MIP-1α and RANTES.

1.4.2.1 MIP-1α (CCL3)

The chemokine MIP-1α was originally described as a macrophage inflammatory protein since it attracts macrophages and usually forms a homodimer or a heterodimer with MIP-1β. The mature proteins of both MIP-1α and MIP-1β are 69 amino acids long (Haelens *et al.*, 1996). It has however, also been shown to attract other cells including DCs, monocytes, NK cells and T-lymphocytes as well as functioning as a stem cell proliferation inhibitor (Uguccioni *et al.*, 1995; Haelens *et al.*, 1996; Rollins, 1997). It has been demonstrated to be an efficient Th1 chemotactic molecule but does not attract Th2 cells (Siveke and Hamann, 1998).

1.4.2.2 RANTES (CCL5)

RANTES is named from 'regulated upon activation, normal T expressed and secreted' and is also a CC chemokine. As this chemokine has been associated with HIV suppression, RANTES has been extensively characterised, although there is still confusion over some of the exact functions of this chemokine. RANTES is thought to be a chemoattractant and activator for cells including monocytes, basophils, eosinophils and T cells and also induces histamine release (Kaplan *et al.*, 1995; Haelens *et al.*, 1996; Rollins, 1997). It has been determined that RANTES is an efficient chemoattractant of Th1 cells but not Th2 cells indicating a role in cell mediated immune responses (Schrum *et al.*, 1996; Siveke and Hamann, 1998). However, Kaplan *et al.*, argue that RANTES also attracts and stimulates eosinophils and basophils causing these cells to release substances such as histamine or eosinophil cationic protein which suggests a role for this chemokine also in the mediation of allergic inflammation, a Th2 response (Kaplan *et al.*, 1995; Haelens *et al.*, 1996).

1.4.3 C chemokines

Two exceptions to the CXC/CC rule are the chemokines single cysteine motif (SCM)-1α (also known as XCL1 or lymphotactin) and SCM-1β (XCL2), characterised by only having two non-adjacent cysteine residues. These two proteins are placed into a new family called the C-chemokines. The genes of both these chemokines map to chromosome 1 at position 1q23 and are both expressed from T lymphocytes and NK cells after activation with IL-2 or IL-12. They share the same receptor, XCR1, and as such are both chemoattractant molecules for NK cells and T-lymphocytes, but not monocytes (Yoshida *et al.*, 1996; Hedrick and Zlotnik, 1998; Hennemann *et al.*, 1999). Other than acting as a chemoattractant during early inflammatory responses, no other function for these chemokines has been identified (Hedrick and Zlotnik, 1998).

1.4.4 CX_3C chemokine

Another exception to the CXC/CC conserved cysteine rule is the CX_3C-chemokine termed fractalkine (CX3CL1, or neurotactin in the mouse), which has 3 amino acids between the first two cysteines. This chemokine is unusual, as it is the first described chemokine that can exist either as a soluble protein or as an integral membrane bound protein expressed on leukocytes. Both forms of fractalkine function predominantly as an

adhesion molecule for leukocytes and cells expressing its receptor, CX_3CR1, in much the same fashion as fibronectin or intracellular adhesion molecule-1 (Rollins, 1997; Cook *et al.*, 2001; Umehara *et al.*, 2001). Fractalkine is also expressed at high levels in neurons and its' receptor is expressed on glial cells in the central nervous system suggesting an important role in glial cell migration. Knockout mice lacking fractalkine however, have no observable abnormalities and their response to various inflammatory stimuli were indistinguishable from those of wild type mice (Cook *et al.*, 2001). This would suggest that fractalkine is not a key chemokine molecule for immune responses. However, it is likely that other chemokines are compensating for the lack of fractalkine in the knockout mice. It is also possible that inappropriate stimuli for fractalkine studies were used since the receptor for fractalkine has been implicated in the progression of AIDS in HIV positive individuals.

1.5 Chemokine Receptors

For chemokines to be effective they must interact with complementary receptors expressed on the surface immune cells. Chemokine receptors form a distinct group of structurally related proteins within the super family of receptors that signal through heterotrimeric GTP-binding proteins (Fig 1.2). All chemokine receptors are approximately 350 amino acids in length consisting of a 7-transmembrane spanning (7-TMS) region and are related by a number of conserved structural motifs, mainly found throughout the 7 transmembrane domains. In addition, all chemokine receptors have two conserved cysteines, one in the NH_2-terminal domain and the other in the third extracellular loop. These cysteines are assumed to form a disulphide bond that is critical for the conformation of the ligand-binding region.

On the basis of the overall sequence identity and the subsequent chemokine for which they bind, four subgroups of receptors have been defined. The CXC chemokine receptors share 33-77% identical amino acids and the CC chemokine receptors share 46-89% identical amino acids (Baggiolini *et al.*, 1997; Murdoch and Finn, 2000). Eighteen chemokine receptors have currently been identified, five of which bind CXC chemokines (CXCR1-CXCR5), ten bind CC chemokines (CCR1-CCR10), one binds fractalkine (CX_3CR1) and one for Lymphotactin (XCR1). The last chemokine receptor of the eighteen is the Duffy antigen receptor for chemokines (DARC) and is expressed in human erythrocytes and on endothelial cells lining post capillary venules in the

spleen and kidneys. DARC binds numerous chemokines as well as acting as a binding protein for the malarial parasite *Plasmodium vivax* (Horuk *et al.*, 1996; Murdoch and Finn, 2000). The sequence of DARC is highly conserved across animal species, and along with its expression on erythrocytes and promiscuous chemokine binding, is thought to act as a chemokine 'sink' to keep chemokine levels in the circulation low (Horuk *et al.*, 1996). The signaling mechanism through which cells respond to chemokine stimuli is not yet well understood, although a number of factors have been shown to be crucial for chemokine receptor signaling. The pathways examined to date have been reviewed by Mellado *et al*, and are summarized in figure 1.2 (Mellado *et al.*, 2001).

Many chemokine receptors bind to multiple chemokine ligands within each respective subfamily (table 1.1) indicating flexibility within the chemokine system that also provides a mechanism for a chemokine gradient, orchestrating complex immune cell movements. However, this myriad of ligand/receptor binding makes elucidating the mechanism whereby specific chemokine receptors and ultimately chemokines function and interact quite difficult (Rollins, 1997). Complexity is also added by the fact that several receptors can be expressed on the same cell type(s) but in different ratios and with different affinities for the same chemokine ligand. For example, studies have been carried out which determined chemokine receptor expression on human Th1 and Th2 cell lines. The procedure involved generating Th1 and Th2 cell lines by stimulating human lymphocytes with either IL-12 plus IL-4 antibody (for Th1 cells) or IL-4 plus IL-12 antibody (for Th2 cells). The Th1/Th2 cell type was confirmed by detecting the production of IFNγ and IL-4 respectively. Expression of various chemokine receptors was detected by mRNA northern blot analysis after activation of the T helper cell types (Bonecchi *et al.*, 1998). A summary of these results can be seen on table 1.2. In a review by Baggiolini *et al.*, additional chemokines are associated with some of the chemokine receptors. The ligands for CCR2 include RANTES, MIP-1α, MCP 1,3, and 4 but not MCP-2. In addition to eotaxin and MCP-3, CCR3 can also bind RANTES. Finally both CCR4 and CCR5 can also bind MIP-1α and RANTES (Baggiolini *et al.*, 1997).

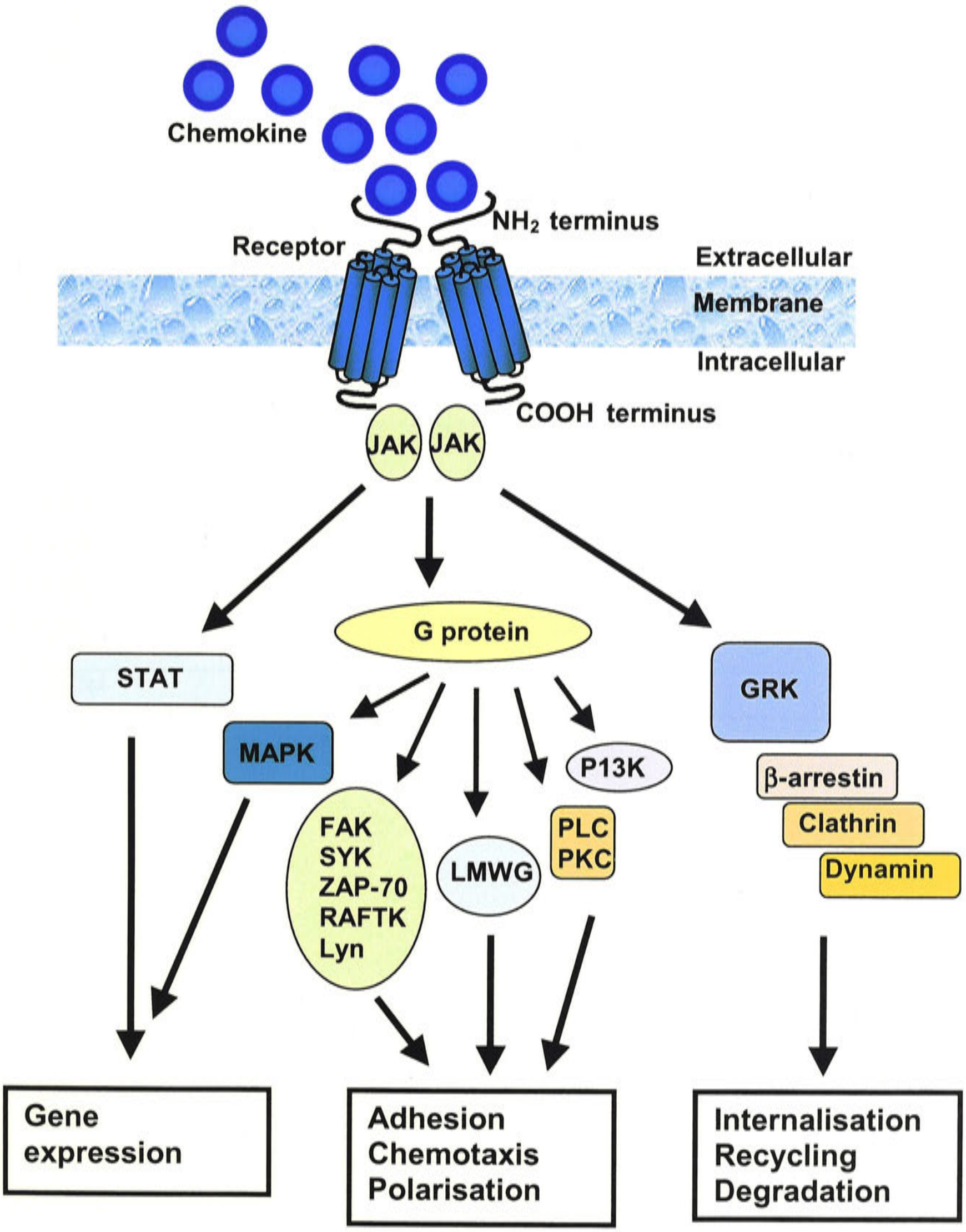


Figure 1.2: Schematic model showing the signalling pathways activated by chemokines binding to their receptors. Selected regulatory molecules and transcription factors are shown. Adapted from (Mellado *et al.*, 2001).

Table 1.2: Summary of chemokine receptor expression on Th1 and Th2 cells. Modified from Bonecchi *et al.*, 1998.

Receptor	Relative expression levels on Th1 and Th2 cells	Points to note
CCR1	Th1 = Th2	
CCR2	Th1 > Th2	Both cell types had relatively high levels of expression
CCR3	Th2 only	Low levels of expression
CCR4	Th1 < Th2	14.6 fold higher expression
CCR5	Th1 > Th2	4.9 fold higher expression
CXCR1	Not detected	
CXCR2	Not detected	
CXCR3	Th1 > Th2	6.7 fold higher expression
CXCR4	Th1 = Th2	

1.6 Diversity of chemokine function

Chemokines have a wide variety of functions with the major purpose being involved in immune cell trafficking or recruitment to sites of infection. Other roles include blood vessel formation (both angiogenesis and angiostasis) and Th1/Th2 lymphocyte differentiation (Rossi and Zlotnik, 2000). As a result of their broad and pivotal roles throughout the body, they have been targeted as proteins with potential therapeutic applications for a variety of diseases.

1.6.1 Immune cell migration

As the name 'chemokine' suggests, derived from *chemo*tactic cyto*kines*, this family of proteins is probably best known for their ability to attract or recruit cells of the immune system to sites of infection. The multiple ligand and receptor binding patterns creates a complex mosaic model for effector cell migration. A cascade model, where chemokines with different affinities for the same receptor initiate a 'chemokine gradient' which cells can follow to the site of interest, is the favoured mechanism for effector cell migration (Foxman *et al.*, 1997). The immune cell has the ability to modify and change receptor expression during the course of maturation and/or activation. Secondary reactions can be initiated by chemokines which induce the expression of cytokines from effector cells and in turn, these cytokines initiate further chemokine expression and activation (Fig 1.3) (Salazar-Mather *et al.*, 2000). Since a large number of chemokines have been identified, it is possible that this may ensure sufficient recruitment of effector cells even if genetic defects or invading pathogens disable the 'normal' migratory chemokine system, whereby a chemokine with an overlapping function may then act to compensate (Mantovani, 1999).

The morphological changes that occur to a migrating cell after initial chemokine activation is reasonably well understood (Sanchez-Madrid and del Pozo, 1999; Mellado *et al.*, 2001). One of the first events to occur when chemokines initially interact with their receptor is receptor dimerization and cellular polarization resulting in two differentiated areas of the cell. There is a redistribution of chemokine receptors to the leading edge of the cell, which is triggered by chemotactic factors as well as cytokines such as IL-15 and IL-2. The redistribution of the chemokine receptors, and also cellular polarization, results in the orientation of the leading edge towards the chemoattractant source. Morphologically, the leukocyte in question will change from a spherical shape

to more of an amoebae-like cell (Fig 1.4) (Baggiolini, 1998). An arm, or leading edge, will form from the cell body followed by a tail-like formation. The cellular polarization causes the pseudopod-like tail to develop, termed a uropod. This represents a specialized structure with important motility and adhesion properties, including the expression of adhesion molecules such as L-selectin, ICAM, Mac-1 and CD43. These molecules promote binding of other cells resulting in enhanced leukocyte recruitment and transendothelial migration (Sanchez-Madrid and del Pozo, 1999; Mellado *et al.*, 2001). The rate at which cells migrate relies on the coordination of the extension and retraction adhesion molecules, for example fibroblasts migrate relatively slowly (1 μm/min) due to a lag time between extension of the leading edge and retraction of the uropod. In contrast T lymphocytes can migrate relatively rapidly (7-7.5 μm/min), due to the extension and retraction forces occurring in a very coordinated manner. This also results in the cell maintaining cell shape and size during movement (Niggemann *et al.*, 1997; Sanchez-Madrid and del Pozo, 1999).

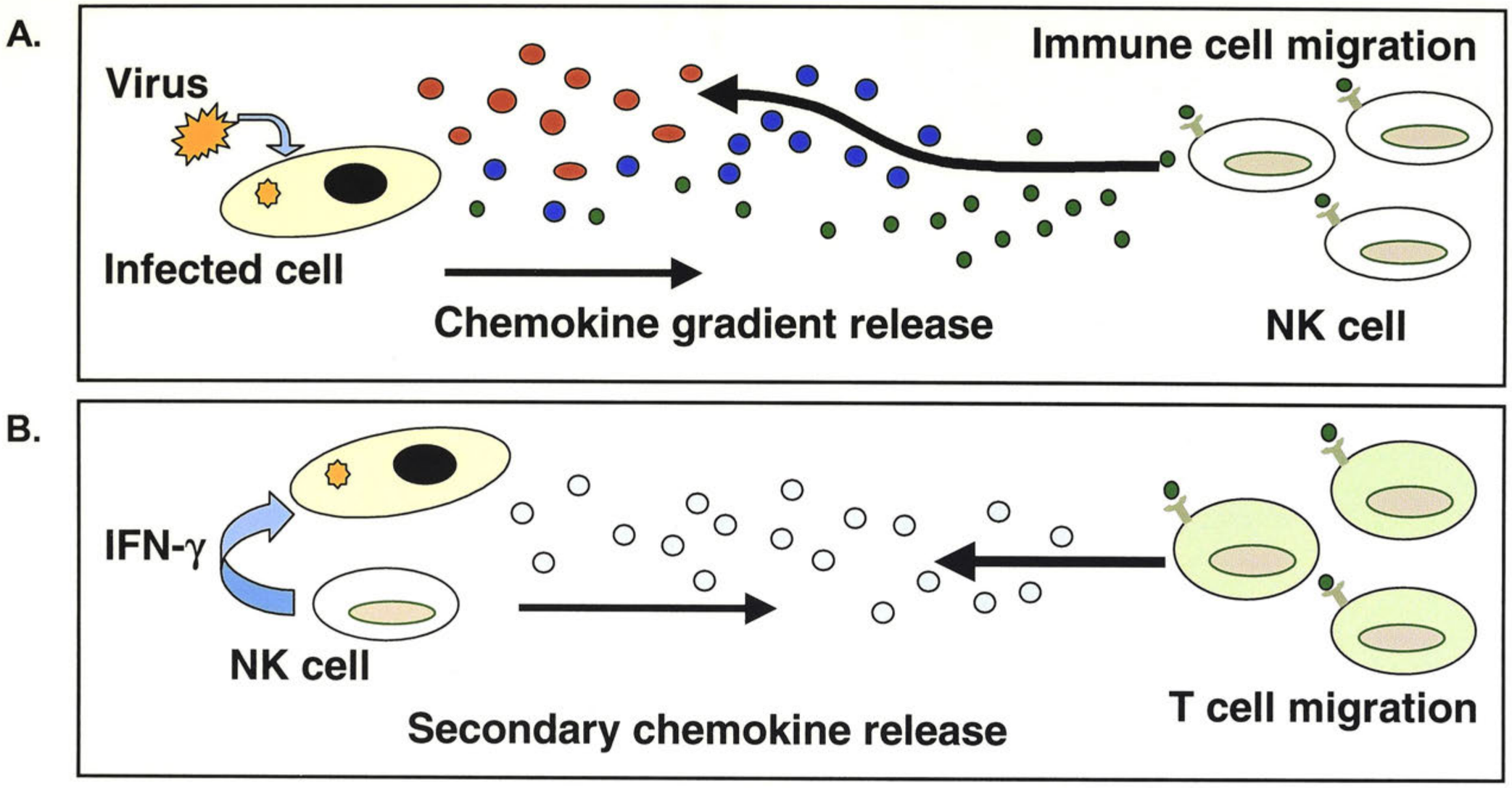


Figure 1.3: Model of migration mechanism. Part A. shows a gradient effect initiated by three different chemokines which the immune cell can 'follow' to the site of infection. Part B. shows the secondary effect when the primary immune cell(s) become activated leading to a secondary release of chemokines by the infected cell and/or NK cell.

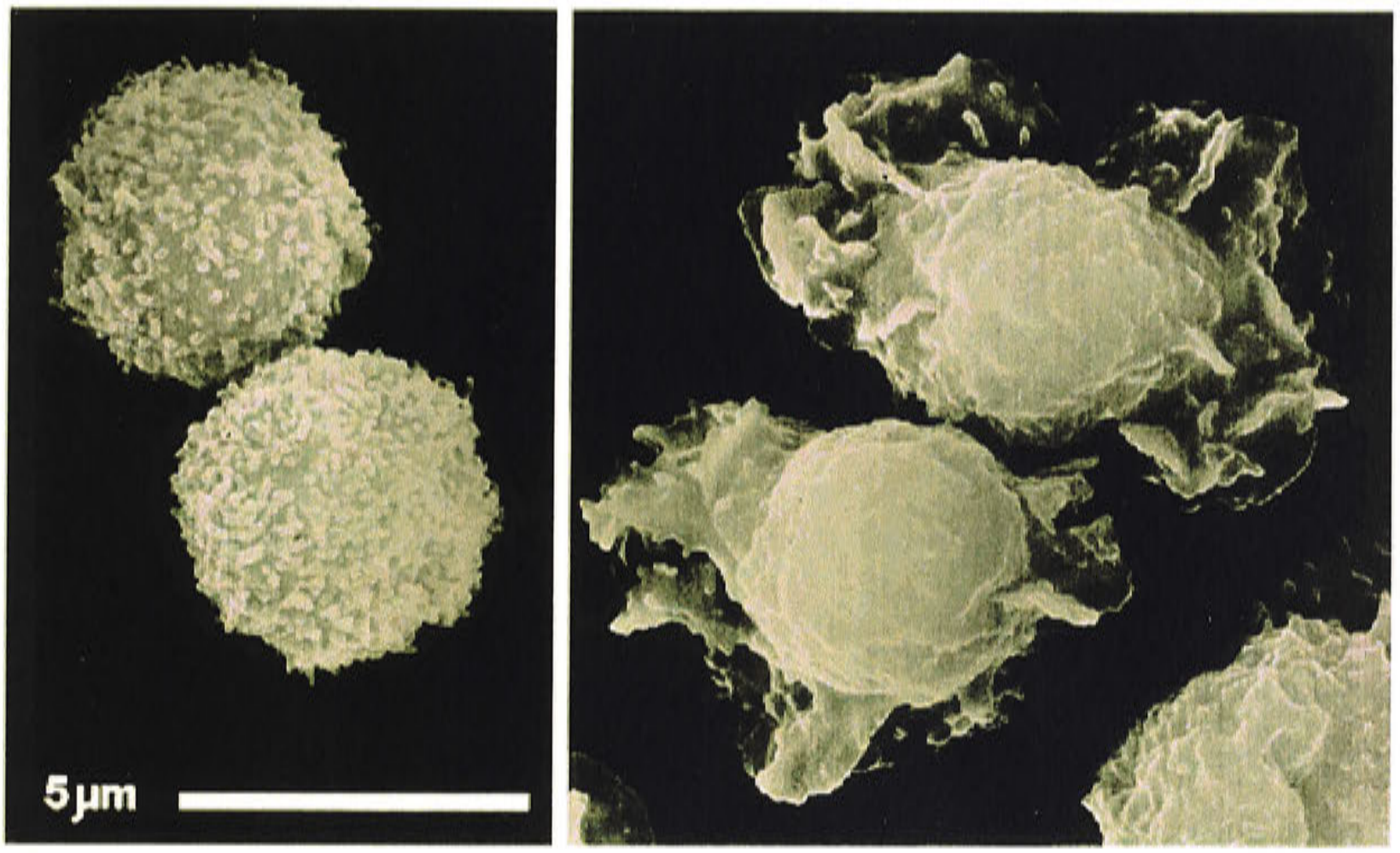


Figure 1.4: Shape change of human neutrophils without chemokine stimulation (left panel) and 5 seconds after stimulation with a chemoattractant (Baggiolini, 1998).

1.6.1.1 DC chemokine and receptor expression

Most cells of the body can express chemokines in order to recruit immune cells to the site of pathogen invasion. However, for cells such as antigen presenting cells, especially DCs, there is a complex interaction between pathogen-infected cells, chemokines, cytokines and other immune cells of the body. DCs migrate to the site of infection, then travel to the draining lymph nodes where they activate the appropriate arm of the immune system. A switch in chemokine and chemokine receptor expression is thought to control this multi-step response (Sallusto *et al.*, 2000; Marquez and Martinez, 2001). Immature DCs express chemokine receptors such as CXCR1, CCR1, CCR2, and CCR5 which bind chemokines that are expressed by infected cells under inflammatory conditions and include IL-8, MIP-1α, RANTES, MCP-1 and MCP-4. While the DC is migrating up the inflammatory chemokine gradient, it is being exposed to cytokines such as TNF-α, IL-1 and bacterial or viral products such as LPS or dsRNA which initiate the maturation of the DC. Once the DC has been exposed to antigen and has become a mature DC, a complete alteration in chemokine receptor expression occurs. Receptors such as CXCR4, CCR4 and CCR7 are all up-regulated while the initial receptors are down-regulated. This enables the DC to then migrate to the lymph node to activate the adaptive immune response (Cyster, 1999; Sallusto *et al.*, 2000; Marquez and Martinez, 2001). Along with chemokine receptor expression switching, a significant change in chemokine expression from DCs also alters during maturation. Moreover, chemokine expression differs, depending on the stimuli that the DC received to mature as well as the effector cells that are required for activation, i.e. a Th1 or Th2 phenotype (Sallusto *et al.*, 1999; Sallusto and Lanzavecchia, 2000).

1.6.1.2 Chemokine potency: chemotaxis assays

Determining the relative hierarchy of those chemokines that attract the same cell or bind to the same receptor can give an indication of a migration cascade that could occur for cellular movement. Chemokine potency can be measured in several ways ranging from intracellular calcium mobilization to cellular migration rates and chemokine ligand-receptor displacement assays.

Chemokine induced cellular migration assays have traditionally been assayed using the Boyden chamber chemotaxis system (Falk *et al.*, 1980). The ability for a chemoattractant molecule to facilitate movement using the Boyden chamber is carried out by adding the cells of interest into an upper chamber, which is separated by a filter from a lower chamber containing the chemoattractant of interest. After a period of incubation, the cells that have migrated through the filter are counted and compared to control samples (Falk *et al.*, 1980). Different concentrations of chemokines can be utilized to obtain an indication of chemokine potency and efficiency. As the chemokine concentration increases, there is enhanced cell movement, both in number and in migration rate, however, there is an optimum chemokine concentration, after which receptors are desensitized resulting in the lowering of migration velocity (Ali *et al.*, 1999).

Recently Heinemann *et al.*, developed a sensitive flow cytometry assay to measure leukocyte shape change as a marker of cell responsiveness. This group examined shape change in basophils in response to a number of different chemokines. Their results showed the ranking of potency of the following chemokines; MCP-4 $\geq$ eotaxin-2 = eotaxin-3 $\geq$ eotaxin > MCP-1 = MCP-3 > MIP-1α > RANTES = MCP-2 = IL-8. The degree of basophil shape change was chemokine concentration and time dependent. These chemokines can also bind to different receptors, and during these studies it was demonstrated that the levels of CCR2 and CCR3 receptor activity altered the chemokine mediated responses (Heinemann *et al.*, 2000). Relative potency of chemokines is not limited to leukocyte migration rates as chemokines themselves can have other functions not related to cellular movement.

1.6.2 Angiogenesis and angiostasis

The formation of new blood vessels, or angiogenesis, is very important for physiological processes including the development of new capillaries during wound repair (Belperio *et al.*, 2000). Several CXC chemokines have been associated with the regulation of angiogenesis. Generally, the ELR containing CXC chemokines, such as IL-8, ENA-78, GRO-α, GRO-β and GRO-γ, which bind to the receptors CXCR1 and/or CXCR2, enhance angiogenesis (Belperio *et al.*, 2000). Their ability to enhance

angiogenesis appears to be a distinct function from their ability to recruit cells during inflammatory responses (Belperio *et al.*, 2000).

In contrast to the enhancement of blood vessel formation, it has been shown that the chemokines PF4, IP-10 and Mig are potent inhibitors of angiogenesis (termed angiostatic chemokines) demonstrating yet another example of the multi-functionality of various chemokines (Angiolillo *et al.*, 1995; Strieter *et al.*, 1995a; Strieter *et al.*, 1995b). It has been observed recently that I-TAC can inhibit neovascularization in the rat corneal micropocket assay of angiogenesis indicating that all three IFN-inducible chemokines (Mig, IP-10 and I-TAC) are potent inhibitors of angiogenesis (Belperio *et al.*, 2000). The mechanism by which these chemokines function is still unclear, although it is thought to involve the inhibition of endothelial cell differentiation into branching networks of tubular structures (Angiolillo *et al.*, 1995).

1.6.3 T lymphocyte differentiation and activation

Cytokines such as IFN-γ and IL-4 have long been known to play an important role in T cell differentiation into Th1 cells or Th2 cells respectively. There is however, emerging evidence that chemokines and their receptors may also have a role in T cell differentiation and subsequent activation from naïve to activated T cells. For example, MCP-1 has been implicated in enhancing T lymphocyte differentiation in the Th2 phenotype direction. Evidence for this is found in mice that lack MCP-1, which have decreased IL-4 production and increased resistance to *Leishmania* infection (Gu *et al.*, 2000). MCP-1 also reduced production of IL-12 by macrophages *in vitro* (Chensue *et al.*, 1996). In addition, *in vitro* studies show that $CD4^+$ T cells which were activated by either T cell receptor cross linking or antigen-pulsed APCs while in the presence of MCP-1 had increased levels of IL-4 (Karpus *et al.*, 1997). There have been two suggested pathways by which MCP-1 could influence T cell differentiation, the first is by the reduction of IL-12 expression by APCs, while the second is the enhanced expression of IL-4 production by activated T cells (Luther and Cyster, 2001). Other MCP chemokines, including MCP-2, MCP-3, and MCP-4 have all been found to decrease IL-12 production *in vitro*, suggesting that they too could influence Th2 differentiation (Braun *et al.*, 2000). With respect to enhancing Th1 differentiation, the chemokines MIP-1α, MIP-1β, and RANTES have all been implicated by either

enhancing IL-12 production, or by directly polarizing Th cells (Luther and Cyster, 2001).

In a similar fashion to DCs, the cells of adaptive immunity also express different chemokines and chemokine receptors depending on the type of immune response they are to elicit as well as their differentiation and activation state(s). For example, naïve T cells express CCR7 and CXCR4, whereas memory Th1 cells up-regulate the expression of CCR1, CCR2, CCR5, and CXCR3, memory Th2 cells up-regulated CCR2, CCR3, and CCR4 expression. Once differentiated and activated, both these memory T cell subsets down-regulated expression of these receptors and up-regulated others including CCR7 and CXCR5 (Sallusto *et al.*, 1999).

1.7 Chemokines and disease; viral infections

As immune cells, cytokines, and chemokines are activated and released to protect the body from disease, it is not unexpected that chemokines and their receptors are an integral part of research into many different diseases. The role of chemokines and pathogen infections, especially viral infections, appears to be the dominant focus of chemokine research. Chemokines and their receptors have several different functions during a viral infection. Virally infected cells can express a number of different chemokines to activate the immune system and aid in the clearance of the virus. From an evolutionary perspective, it is hardly surprising that viruses themselves have developed methods to try and subvert the chemokine antiviral system. Viruses can encode chemokine or receptor homologues that can aid in their evasion of the immune response and, as in the case of HIV, viruses can use chemokine receptors as co-receptors necessary for viral entry.

1.7.1 Chemokine expression initiated by viral infections

Cells infected with virus release a myriad of chemokines in order to activate the immune system to fight infection. Research into the expression of the chemokines released during infection not only aids in the understanding of viral pathogenesis, but it also aids the elucidation of the mechanisms the chemokine system uses to overcome these infections. The expression of each chemokine is dependent on the type of viral infection as well as the progression and state of both viral infection and immune response. For example, Matikainen *et al.*, carried out a study examining chemokine

expression from macrophages infected with either influenza A virus or Sendai virus. Both viruses cause a number of different chemokines to be expressed including MIP-1α, RANTES, MCP-1, MCP-3, and IP-10. The levels of expression of these chemokines however, differ between the two viruses as well as the time points at which peak expression is detected. Some other chemokines are expressed in one virus model and not the other, for example Sendai virus induces expression of IL-8 whereas influenza A does not (Matikainen *et al.*, 2000).

Another example of two chemokines being expressed at different stages during a viral disease, and therefore having different functions was demonstrated by Salazar-Mather *et al.* This study demonstrated that both MIP-1α and Mig chemokine expression is important in viral clearance in mice infected with murine cytomegalovirus (MCMV). During a MCMV infection, initial expression of the CC chemokine MIP-1α promotes NK cell inflammation in the liver. The presence of NK cells contributes to defense against MCMV infections through the production of IFN-γ. It was reported that peak liver expression of Mig is dependent upon the presence of MIP-1α, NK cells, and IFN-γ. Mig then mediates an antiviral role through the recruitment of more effector cells such as T lymphocytes to sites of infection (Salazar-Mather *et al.*, 1998; Salazar-Mather *et al.*, 2000).

1.7.2 Viral evasion mechanisms

As cytokines and chemokines are crucial for an effective innate and adaptive immune response, these molecules are often the targets for immune avoidance mechanisms developed by many pathogens, particularly viruses (Spriggs and Sher, 1999). For example, several poxviruses including cowpox, variola (smallpox), EV and myxoma (rabbit pox) viruses encode proteins that have chemokine, cytokine and receptor homology (Lalani *et al.*, 2000). There are three common strategies that viruses have developed to modulate chemokine activity. These include (i) the production of molecules which mimic chemokine functions, (ii) the expression of chemokine receptor homologues, which presumably compete for chemokine binding, and (iii) the production of soluble chemokine-binding proteins to inhibit host chemokine activity (Lalani *et al.*, 2000).

An example of virus encoded chemokine homologues is found in molluscum contagiosum virus (MCV), which is a poxvirus that causes benign tumours in the skin of immunocompetent children. MCV encodes a 104 amino acid genome sequence (MC148R) that has broad homology to the CC family of chemokines, and is most related to the cellular chemokine IL-11-receptor-α-locus (ILC, also known as CTAK or CCL27). The exact role that MC148R has *in vivo* is unclear, however it is secreted from mammalian cells *in vivo*, and it has been predicted that the protein is involved in inflammatory responses (Bugert *et al.*, 1998; Lusso, 2000).

Human Kaposisarcoma virus, or HHV-8, also encoded chemokine homologues, including vMIP-1, vMIP-II, and vMIP-III, which act to bind the human receptors CCR8, CCR3, and CCR4 respectively (Boshoff *et al.*, 1997; Dairaghi *et al.*, 1999; Stine *et al.*, 2000). As these receptors are linked to Th2 responses, it has been suggested that the expression of these chemokine homologues, drive the immune response towards a Th2 response, thereby hindering the antiviral Th1 response (Lusso, 2000).

Several viruses encode chemokine receptor homologues, which may function as chemokine agonists. By binding circulating chemokines without initiating intracellular antiviral signal, the virus causes the chemokine to be ineffective. It is however, still unclear how the functional mechanisms of these virus-encoded proteins operate (Mahalingam and Karupiah, 2000b). Virus encoded receptor homologues generally have broad spectrum chemokine binding, for example, the human cytomegalovirus (CMV), has a gene product (US28) which binds a large variety of CC chemokines including MIP-1α, MCP-1 and RANTES (Neote *et al.*, 1993). Another example is the herpesvirus saimiri (HVS) gene product (ECRF3) which can bind multiple CXC chemokines, including IL-8, Gro-α, β, and γ as well as NAP-2 (Ahuja and Murphy, 1993).

The third strategy by which viruses use the chemokine system to evade the immune system is the expression of chemokine-binding proteins, which act as chemokine scavengers. These proteins, which are expressed by several viruses, are not related to chemokines or their receptors, in that they do not share any sequence similarity (Mahalingam and Karupiah, 2000b). An example of a chemokine binding protein is

found in the Lister strain of VV, which secretes a 35 kDa protein that inhibits eotaxin function. This chemokine binding protein also binds other CC chemokines including RANTES, MIP-1α, MCP-1, and I-309 (Alcami *et al.*, 1998). The discovery of proteins such as these may be beneficial for therapeutic use. For example, the ability of this binding protein to block eotaxin may be used to inhibit eosinophil infiltration during allergic inflammatory reactions (Alcami *et al.*, 1998).

1.7.3 Viral entry using chemokine receptors

Binding of virus particles to the host cell surface initiates a cascade of events that allows entry of the genetic material of the virus into the cell. An example of a virus gaining entry into cells by using chemokine receptors is the human immuno-deficiency virus (HIV). It has been proposed that, for HIV-1, receptor recognition may serve as a trigger that initiates entry. Although CD4 was identified as the primary receptor for HIV-1, an additional co-receptor was necessary for infection (Bates, 1996). The co-receptor (for entry of T-tropic HIV-1 strains) was isolated and initially termed 'Fusin' and was closely related to the IL-8 receptor CXCR2 (Feng *et al.*, 1996). Fusin was later found to be the receptor for the chemokine SDF-1 and renamed CXCR4 (Bleul *et al.*, 1996; Oberlin *et al.*, 1996). The receptor CCR5 was also identified to be a co-receptor for HIV-1 (M-tropic virus strains) (Alkhatib *et al.*, 1996; Dragic *et al.*, 1996). Cocchi *et al.*, have shown evidence that RANTES, MIP-1α, and MIP-1β are major HIV-suppressive factors produced by $CD8^+$ T cells. This is most likely due to competition for the chemokine receptors (Cocchi *et al.*, 1995).

A 32 base pair deletion in the second extracellular loop of the CCR5 gene was identified and was found to cause a frame-shift and premature truncation of the polypeptide (Liu *et al.*, 1996). Individuals who are homozygous for the mutation are highly resistant (but not immune) to virus infection, while patients with heterozygous products have a slower progression of the disease than do normal patients (Liu *et al.*, 1996; Samson *et al.*, 1996; Doms and Peiper, 1997). Interestingly the frequency of this mutation is surprisingly high in the western European population with about 1% of Caucasians being homozygous, and 18% heterozygous (Samson *et al.*, 1996). This suggests that there has been some selective pressure in the past, perhaps from another infectious

agent, which gave individuals with this allele a survival advantage (Doms and Peiper, 1997).

1.8 Chemokines and other diseases

Not only can chemokines recruit cells to sites of pathogen infection to aid recovery, they can also recruit inflammatory cells to sites where their benefits are not so well understood and in fact, may be harmful to the host. Diseases such as multiple sclerosis, allogenic transplant rejection, asthma and tumour progression are all regulated by the presence and activity of chemokines and their receptors.

1.8.1 Autoimmunity - Multiple sclerosis and EAE

There have been numerous studies examining the role of several chemokines during autoimmune diseases such as multiple sclerosis and a similar mouse model, experimental autoimmune encephalomyelitis (EAE). EAE is a CNS demyelinating disease induced in susceptible mice, rats, guinea pigs, or non-human primates following immunization with myelin derived antigens, peptides or following transfer of autoreactive CD4+ T cell lines or clones (Willenborg *et al.*, 1996). During the initiation of EAE, activated Th1 autoreactive T cells appear to infiltrate the CNS by an antigen independent and chemokine independent process which leads to the increased expression of a range of proinflammatory cytokines within the CNS (Hickey *et al.*, 1991; Glabinski *et al.*, 1995). These cytokines then, among other effects, upregulate the expression of a suite of chemokines which have been implicated in various stages of EAE disease. Some of the chemokines expressed during various stages of EAE include RANTES, MIP-1α, MIP-1β, MCP-1 and IP-10. (Hulkower *et al.*, 1993; Ransohoff *et al.*, 1993; Glabinski *et al.*, 1995; Godiska *et al.*, 1995; Glabinski *et al.*, 1997; Youssef *et al.*, 1998; Glabinski and Ransohoff, 1999). Due to the overlapping roles of chemokines and chemokine receptors, the exact role of each chemokine in disease progression is unclear even when utilizing knockout mice for various receptors or chemokines (Gerard and Rollins, 2001).

1.8.2 Asthma

Studying of the function of RANTES during allergy, predominantly thought of as a Th2 type disease, provides an example of the difficulty in elucidating the exact function of various chemokines in disease. John *et al.*, carried out a study investigating RANTES, a strong chemoattractant molecule for eosinophils and monocytes in human airway smooth muscle during allergic inflammation. This group demonstrated that, *in vitro*, RANTES mRNA expression and subsequent protein expression was induced independently by TNF-α but not IFN-γ (both Th1 cytokines). However the combination of IFN-γ and TNF-α caused a synergistic degree of expression and release in both a time and dose dependent manner. RANTES production was partially inhibited by the Th2 derived cytokines IL-4, IL-10, and IL-13. It has been proposed that RANTES and possibly other chemokines may participate in chronic airway inflammation by interacting with both Th-1 and Th-2 derived cytokines to modulate chemoattractant activity for eosinophils, activated T lymphocytes, monocytes, and macrophages (John *et al.*, 1997). An experiment carried out by Gonzalo *et al.*, using mouse eotaxin (a chemoattractant for eosinophils during allergic reactions) demonstrated that Th1 cell clones expressed eotaxin to levels similar to that of Th2 cell clones. These findings were further confirmed in an *in vivo* situation where the Th2 response is impaired. IL-4 deficient mice (and IL-5 levels are reduced by 70-90%) are still able to express eotaxin, in the lungs, in similar levels to wild type mice (Ganzalo *et al.*, 1996).

1.8.3 Transplantation

Chemokines have also been implicated in the rejection of transplanted grafts through their capacity to elicit immune cell migration. The exact mechanism by which transplantation rejection occurs is unclear. Migration of immune cells such as T lymphocytes, macrophages, NK cells, DCs and other APCs however, is thought to be involved as rejection occurs after those cells have migrated either to or from the transplant site and lymphoid organs and have recognized the material as foreign. In fact, the migration of DCs from the graft to secondary lymphoid organs is of primary significance to the rejection process (Lakkis *et al.*, 2000). Like EAE and other autoimmune diseases, numerous chemokines are expressed during the rejection process, and again, due to the overlapping roles of these chemokines, the exact function of each chemokine in the transplant process is difficult to elucidate and control (Nelson and

Krensky, 2001). However, Hancock *et al.*, have demonstrated that the IFN-inducible chemokines, Mig and IP-10 are expressed at high levels during acute cardiac allograft rejection, and CXCR3 deficient mice show profound resistance in the development of transplant rejection (Hancock *et al.*, 2000).

1.8.4 Tumour progression

One reason why the study of chemokines during angiogenesis and angiostasis is important is that many tumours require a good blood vessel network for successful growth (Belperio *et al.*, 2000). Therefore, the *in vivo* inhibition of angiogenic chemokines, the ELR-CXC chemokines, or the enhancement of angiostatic chemokines, such as IP-10, Mig, and I-TAC, or their receptor, CXCR3, may be beneficial in the control of tumours. Indeed, the addition of IP-10, Mig, and/or I-TAC to primary cultures of human microvascular endothelial cells, which express the CXCR3 receptor during the S/G2-M cell cycle phase inhibit the proliferation of this cell line. This effect could be reversed by the addition of anti-CXCR3 antibody (Romagnani *et al.*, 2001a).

Sgasari *et al.*, have carried out a study injecting human Mig into Burkitt's lymphoma which were growing subcutaneously in nude mice. Central and homogeneous tissue necrosis was observed around and within the tumour mass demonstrating antitumour activity of Mig (Sgadari *et al.*, 1997). The exact mechanism for this occurring is unclear although it is unlikely to use T cell or NK cell activity, which are recruited by Mig. It has been suggested that Mig enhances vascular damage associated with elastin destruction and intravascular thrombosis (Sgadari *et al.*, 1997; Kanegane *et al.*, 1998).

1.9 Chemokines for medical use

The multitude of chemokines and the different functions they carry out during an immune response has motivated researchers to question how the chemokine system can be utilized for therapeutic intervention. There are several potential mechanisms by which this could occur. Blocking chemokines or their receptors could reduce inflammation, which may be useful during such autoimmune diseases like multiple sclerosis. Enhancing expression of specific chemokines could alter the type of immune response, either directly or by inhibiting or enhancing other regulatory factors. Incorporating chemokines into vaccines could attract the correct type of immune cells to

fight a specific pathogen, thereby making the vaccine more efficient. Several areas are now being examined that can utilize these molecules in the treatment of disease.

In a study examining vaccinations with either a fusion protein or DNA constructs encoding a fusion protein between a previously non-immunogenic tumour antigen and either IP-10 or MCP-3 generated protection against a B cell lymphoma challenge (Biragyn *et al.*, 1999). Constructs of IP-10 or MCP-3 fused to lymphoma immunoglobulin (Ig) variable regions (sFv) were produced to form either a DNA vaccine or a recombinant fusion protein. The recombinant proteins retained conformation of the native lymphoma derived Ig as well as functional chemotactic properties. Mice were challenged with appropriate tumour cells two weeks after their respective immunization regimes. The fusion protein vaccines elicited inflammatory responses *in vivo*, however mice vaccinated with the DNA constructs generated significant protection against large tumour challenges. This difference is most likely due to the ability of a DNA vaccine to enhance $CD8^+$ mediated immune responses (Biragyn *et al.*, 1999).

An example of enhancing chemokine expression during a disease model was demonstrated by Narvaiza *et al.*, who examined the anti-tumoural effect that IP-10 and IL-12 had on subcutaneous murine tumour nodules derived from the CT26 murine colorectal adenocarcinoma cell line. During this study, two adenoviruses, one encoding IP-10, the other IL-12, were co-localized at the same tumour nodule(s). When used separately, the adenoviruses did not completely clear the tumours, with IP-10 showing some anti-tumour activity, and IL-12 about 70% tumour clearance. However, when used together, 100% of tumours were eradicated by this method, showing a synergistic effect (Narvaiza *et al.*, 2000).

In addition to studies specifically targeting the chemokine system for therapeutic applications, some drug treatments that were previously unknown to affect the chemokine system are already in use. Drug treatment of the chronic inflammatory skin disease, psoriasis, with the use of fumaric acid esters such as dimethylfumarate (DMF) has been examined in human trials for several years (Nieboer *et al.*, 1990; Kolbach and Nieboer, 1992; Mrowietz *et al.*, 1998). However the mechanism by which DMF reduces the symptoms of disease has, until recently, remained elusive. It has been shown that

DMF inhibits the expression of several chemokines including, GRO-α, IL-8. Mig, IP-10 and HuI-TAC from keratinocytes and peripheral blood mononuclear cells in a dose dependent manner (Stoof *et al.*, 2001). This inhibition of chemokine expression interferes with the recruitment of T lymphocytes and other inflammatory cells in the dermal and epidermal compartments of the skin (Stoof *et al.*, 2001).

1.10 Scope of this thesis

Identifying and characterising the chemokines expressed by the body is crucial for understanding the immune system as a whole, as well as the mechanisms utilized in the battle against disease. In addition to this, understanding chemokine function during an immune response will have important benefits for designing vaccine and therapeutic techniques to help overcome many diseases. Characterizing murine homologues of human chemokines is an invaluable resource to further the understanding of how chemokines function *in vivo* and during different diseases.

The research presented here describes the characterisation of the murine homologue to human I-TAC, which was isolated from a dendritic cell line after stimulation with IFN-γ. Several approaches were taken to characterize this chemokine, including sequence analysis, *in vitro* and *in vivo* expression studies, as well as functional analysis, and using I-TAC itself to modify immune responses. The cDNA sequence verifies that it is a non-ELR CXC chemokine homologous to MuMig and Crg-2. Producing the I-TAC protein and using it in a chemotaxis assay demonstrates that it is a functional chemoattractant molecule. The cytokines involved in inducing I-TAC expression was analyzed by stimulating two DC cell lines with various cytokines and other factors. To look at the *in vivo* mechanism for I-TAC induction, various knockout mice were utilized. After finding that IFN-γ primarily induces I-TAC *in vivo* and from dendritic cells, several murine disease models were also examined to determine whether or not this chemokine is expressed during immune responses against these diseases. The different disease models include three virus models, these being influenza, respiratory syncytial virus (RSV) and VV, as well as the autoimmune disease model, experimental autoimmune encephalomyelitis (EAE) and two transplantation models. The potential for artificial manipulation of I-TAC for medical applications, such as in a vaccine situation, was also

assessed. A recombinant VV encoding I-TAC was constructed and various experiments carried out to elucidate the role of I-TAC during a rVV infection.

CHAPTER 2.

ISOLATION AND CHARACTERISATION OF MURINE I-TAC

CHAPTER 2. ISOLATION AND CHARACTERISATION OF MURINE I-TAC

2.1 Introduction

The mature human I-TAC protein is a non-ELR CXC chemokine and consists of 94 amino acids, is homologous to human Mig and IP-10, and binds to the CXCR3 receptor. (Rani *et al.*, 1996; Jacobs *et al.*, 1997; Cole *et al.*, 1998). Human I-TAC is a potent chemoattractant for activated T lymphocytes, NK cells and monocyte-like cells (Cole *et al.*, 1998; Luo *et al.*, 1998; Gasperini *et al.*, 1999). It was demonstrated recently that human I-TAC specifically attracts T cell receptor (TCR) $\alpha\beta^+CD8^+$ single positive T cells, TCR $\gamma\delta^+$ T cells, and NK-type cells in human thymus (Romagnani *et al.*, 2001b). The expression of human I-TAC has been detected in a number of different cell types including cultured primary monocytes, cultured fetal astrocytes, a microglial cell line, astrocytoma cells, atheroma-associated cells, bronchial epithelial cells, polymorphonuclear neutrophils and keratinocytes (Rani *et al.*, 1996; Cole *et al.*, 1998; Gasperini *et al.*, 1999; Mach *et al.*, 1999; Sauty *et al.*, 1999; Tensen *et al.*, 1999b). Moderate expression has also been detected in various tissues including the central nervous system (CNS), pancreas, lung, thymus and spleen (Cole *et al.*, 1998; Luo *et al.*, 1998). *In vitro* studies have demonstrated that expression of human I-TAC appears to be up-regulated following exposure to mainly IFN-α, IFN-β or IFN-γ plus other cell regulators such as IL-1, LPS or TNF-α (Rani *et al.*, 1996; Cole *et al.*, 1998). For example, stimulation of astrocytes with IFN-γ and IL-1 results in a 400,000 fold increase in human I-TAC mRNA expression (Cole *et al.*, 1998). The expression of this chemokine by antigen presenting cells (APCs) to recruit activated T cells suggests that human I-TAC has an important role in the integration between the innate and adaptive immune system.

2.1.1 APCs; the dendritic cell

As APCs are an important mediator between the innate and adaptive immune response, many studies examining the role of chemokines during this process have been carried out previously (reviewed in Cyster, 1999; Sallusto *et al.*, 2000; Marquez and Martinez, 2001). Fully understanding this system may enable the manipulation of the immune response for therapeutic applications. The most potent APC in the immune system is the

DC, which can present antigen as well as activate naïve $CD4^+$ T cells *in vivo* (Levin *et al.*, 1993). Characterisation of DC lines has an important role in the elucidation of intracellular activities and expression of immune molecules, including chemokines, which aid the study of immune cell interactions (MacKay and Moore, 1997). Producing an immortalized cell line has an added advantage over a non-immortalized cell line, as homogeneous cell populations can be continuously cultured that respond uniformly to given stimuli (MacKay and Moore, 1997). DC lines such as MTHC-D2 and JAWS II are uncommitted progenitor DCs that can be stimulated to mature, differentiate, and become activated when exposed to different cytokines and stimulatory factors.

2.1.1.1 The MTHC-D2 line

The Myb-transformed hematopoietic cell line (MTHC-D2) is a cloned cell line derived from 12-day fetal liver which was then transformed with a truncated form of the transcription factor, c-myb, which regulates cell proliferation (Gonda *et al.*, 1993; Banyer and Hapel, 1999). This parental cell line was initially described as having characteristics of monocyte-like cells that can then differentiate into macrophage-like cells upon stimulation with IL-4 or dendritic-like cells after stimulation with IFN-γ (Banyer and Hapel, 1999). However, since the discovery of different DC subtypes, this cell line is thought to represent a progenitor DC line that can differentiate into DCs with cell mediated immune response (CMI) properties after stimulation with IFN-γ or DCs with more phagocytic properties after IL-4 simulation (Banyer *et al.*, 2002 submitted, see appendix 1c).

2.1.1.2 The JAWS II line

The JAWS II DC line was developed in a different fashion from the MTHC-D2 line and therefore is a useful alternative in expression analysis. The JAWS II dendritic cell line was isolated from bone marrow tissue collected from p53 knockout mice (MacKay and Moore, 1997). The protein p53 is a growth suppressor factor known to be involved in regulating cell division and growth thereby enabling the JAWS II DC line to be immortalized (MacKay and Moore, 1997). The JAWS II line may be induced to mature by cytokine stimulation, which stimulates antigen presentation and T cell proliferation (MacKay and Moore, 1997). JAWS II cells have been characterised as DCs due to their

morphological attributes, as well as the expression of various differentiation markers including; Mac-1, Fcγ receptor, MHC class I and II, CD44 and intercellular adhesion molecule-1 (ICAM-1) (MacKay and Moore, 1997).

Identifying the murine homologue of human I-TAC is crucial for further, rapid characterisation of this chemokine. This would enable the use of murine models to thoroughly investigate the role of this chemokine *in vivo*, as well as an opportunity to use I-TAC to manipulate the immune system to assess I-TACs' potential use in vaccines and therapies. This chapter describes the identification and preliminary characterisation of the murine chemokine I-TAC after being detected and isolated by a cDNA subtraction technique from the MTHC-D2 DC line.

2.2 Materials and Methods

2.2.1 Mice

Female C57BL/6 mice aged six to eight weeks were obtained from the Animal Breeding Establishment (ABE), John Curtin School of Medical Research (JCSMR), Canberra, Australia.

2.2.2 DC Cell lines

The semi adherent DC progenitor cell line, MTHC-D2, was subcloned from MTHC-D2 lines described previously (Gonda *et al.*, 1993; Banyer and Hapel, 1999). This cell line was maintained in Iscove's modified eagle medium (IMEM; GIBCO/BRL) supplemented with 10% FBS (GIBCO/BRL), 2mM glutamine (SIGMA), and 50 U/mL GM-CSF (Baculovirus produced, JCSMR). The adherent progenitor DC cell line, JAWS II (ATCC, CRL-11904), isolated from $p53^{-/-}$ mice, was maintained in alpha minimum essential medium with ribonucleosides and deoxyribonucleosides containing L-glutamine (GIBCO/BRL) and supplemented with 20% fetal bovine serum (FBS) (Gibco/BRL), 1mM sodium pyruvate and 5ng/mL murine GM-CSF (Baculovirus produced, JCSMR). Both the JAWS II and MTHC-D2 cell lines were stimulated for 48 hrs with GM-CSF (5 U/mL) with or without TNF-α (500 U/mL, Baculovirus produced, JCSMR) and IFN-γ (2 U/mL, Baculovirus produced, JCSMR).

2.2.3 Oligonucleotide Primers

Oligonucleotides were synthesized and supplied at a concentration of 0.2 µmole contained in ammonium hydroxide (Biomolecular Resource Facility, Centre for Molecular Structure and Function, JCSMR). To prepare primers, 100µL aliquots were added to 1mL of n-butanol and centrifuged (14k rpm x 10 min). The supernatant was removed, pellets dried (37°C @ 10 min), resuspended in milli-Q water (MQW), and the concentration determined by UV absorbance at 260nm. Primer concentrations were then adjusted to a standard polymerase chain reaction (PCR) concentration of 20 µM.

2.2.4 Sequencing

Sequencing was carried out using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and processed on an ABI Prism 377 DNA

automated sequencer (PE Applied Biosystems) by the biomolecular resource facility at JCSMR.

2.2.5 PCR and semi-quantitative RT-PCR

Polymerase chain reactions (PCR) were carried out using an Omnigene PCR thermocycler (Hybaid Limited, UK) and thermostable DNA polymerase purified from bacteria which expressed *T. aquaticus* DNA polymerase (Taq polymerase) (Engelke *et al.*, 1990). In addition to Taq polymerase (approximately 1.5 units per 20µL reaction), other reaction components were, 1X PCR buffer (10mM tris[hydroxymethyl]-amino methane (Tris) chloride pH 8.3, 50mM KCl, and 1mg/mL gelatin), 2mM $MgCl_2$, 0.2mM dNTPs (dATP, dCTP, dGTP, dTTP), primers (2µM). Reaction volumes were generally 10µL or 20µL, in 250µL thin walled tubes. PCR reactions were started (hot start) by the addition of Taq polymerase during the first denaturation step at 94°C. The thermal profile used for I-TAC was as follows: Denaturing at 94°C for 10s, annealing at 66°C for 20s, and extension at 72°C for 30s for 30 cycles. The oligonucleotide primers that amplify a 252 bp I-TAC fragment were, mITAC sense, 5'-GAACAGGAAGGTCACAGCCATAGC-3' and mITAC antisense, 5'-ATGAGGCGAGCCTGCTTGGATCTG-3'. All results were standardized against murine GAPDH by amplifying a 551 bp fragment using the primers mGAPDH sense, 5'-ACCACAGTCCATGCCATCAC-3' and mGAPDH antisense, 5'-TCCACCACCCTGTTGCTGTA-3'. The thermal profile used to amplify the GAPDH fragment was as follows: Denaturing at 94°C for 10s, annealing at 58°C for 20s, and extension at 72°C for 30s for 28 cycles. RT-PCR was carried out using 1-2 µL of first strand cDNA (section 2.2.13) in the PCR reaction mixture (above). Prior to the actual assays the thermal conditions and number of cycles used were optimized for both primer pairs so that the PCR and RT-PCR products obtained did not proceed past the linear phase of the reaction nor exceed analysis limits. PCR reactions were visualized as described in section 2.2.6. PCRs and RT-PCRs were repeated three times to ensure reproducibility.

2.2.6 Restriction Enzyme Digests and Agarose Gel Electrophoresis

DNA was digested with commercially supplied restriction enzymes (New England Biolabs (NEB), USA) in the supplied buffers using the suggested protocols. Restriction

digests were stopped by the addition of loading buffer (1x stain [0.025% bromophenol blue and 1.5% Ficoll 400 in MQW], 15mM Tris chloride pH 7.8, 50% [v/v] glycerol and 7mM EDTA in MQW). This buffer was also added to PCR reactions (section 2.2.5) prior to loading onto agarose gels. Restriction digested DNA was separated by agarose gel electrophoresis and visualized using UV fluorescence. Agarose gels were made with NuSieve GTC agarose (BioWhittaker Molecular Applications, USA), generally 3% [w/v] and 1.25µg/ml ETBr in TAE buffer (40mM Tris acetate pH 8.5, 2mM EDTA in MQW). Gels were run in TAE buffer at 80 volts. DNA size standards used for agarose gels was Sau3AI restricted pUC18 plasmid DNA. Images were taken using the NovaLines gel documentation system under UV light.

2.2.7 Plasmid Cloning

DNA plasmids were constructed using a range of different restriction enzymes and sources of insert DNA. Briefly, plasmid vector DNA (5-10µg) was restriction digested in a total volume of 100µL overnight at 37°C and purified by two washes of 100µL phenol/chloroform (25:24:1 phenol, chloroform, isoamyl alcohol, Sigma), and one wash of 100 µL chloroform. The vector was then dephosphorylated using shrimp alkaline phosphatase (Boehringer Mannheim) using 1µL vector per 10µL total phosphatase reaction, incubated at 37°C for 15 min then at 65°C for 15 min to heat inactivate the enzyme, as described in the supplied protocol.

Insert DNA used for plasmid cloning was either PCR generated or isolated from other DNA plasmids by restriction digestion. PCR and restriction digested fragments separated by agarose gel (section 2.2.6) were recovered by centrifuging excised bands in two eppendorf tubes. The top tube had a Whatman 3MM paper cup and a hole in the bottom. The tubes were spun in an eppendorf centrifuge (14k rpm x 30s) with DNA collecting in the bottom tube. The resulting solution was used directly for ligation reactions. The ligation reaction contained 10µL dephosphorylated vector solution (see above), 7µL insert, 2µL 10x ligation buffer (NEB), and 0.5µL or 1 µL of T4 DNA ligase (NEB) depending on whether the fragments had cohesive or blunt ends respectively. Ligation reactions were incubated (16°C x 1 hr) then 1/10 of the reaction mix was transformed into electrocompetent DH10B^{TM} cells (GibcoBRL) using the supplied protocol. Transformed bacteria were incubated in 2mL L-broth (37°C x 1hr),

then 100 µL was spread onto L-agar plates (10g NaCl, 5g yeast extract, 10g tryptophan make up to final concentration of 15g/L) which generally contained the antibiotic ampicillin (~50µg/mL) and incubated (37°C) overnight. Bacterial colonies containing plasmid DNA were detected by one of three methods: 1) blue/white selection, which detects that insert DNA has been cloned into the β-galactosidase operon of some plasmids (e.g., Bluescript II KS$^-$, Stratagene, USA). The enzymatic colour reaction for blue/white selection required isopropylthio-β-galactoside (4µL of a 100mM stock per L-agar plate) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (40µL of a 2% [w/v] stock per L-agar plate) to be spread on L-agar plates prior to the addition of transformed bacteria. 2) Restriction digest of minipreped plasmid DNA (section 2.2.8) and 3) PCR screening which involved transferring single colonies directly into PCR reactions after replicaplating. Correctly cloned plasmid DNA was further checked by minipreping and sequencing (sections 2.2.8 and 2.2.4). Stocks of transformed bacteria were stored in glycerol stock solutions (20-30% glycerol) at -70°C.

2.2.8 Plasmid preparation and Minipreps

Transformed *E. coli* bacteria were cultured in L-broth (10g Bacto-tryptone, 5g bacto-yeast extract and 10g NaCl per L and autoclaved) overnight at 37°C. Miniprep plasmid DNA was prepared from overnight bacteria cultures using a modified alkaline lysis method (Birnboim and Doly, 1979). Briefly, 1.5 mL of culture was placed in eppendorf tubes, centrifuged (14k rpm x 1 min), and supernatant removed. Cells were then resuspended by vortexing in 200µl solution 1 (50mM glucose, 10mM EDTA, 250mM Tris, pH 8.0 made up with MQW and store at 4°C), and left at room temperature for 5 min. 400µL freshly made solution 2 (1% SDS, 0.2M NaOH, made up with MQW) was added, tubes inverted 4 times, and left on ice (5 min). 300µL solution 3 (3M potassium, 5M acetate, made up with MQW) was added, tubes inverted to mix thoroughly, and left on ice (5 min). Tubes were then centrifuged (14k rpm x 3 min) and 800µL of supernatant collected and placed into sterile eppendorf tubes. 500µL isopropanol was then added, tubes vortexed and left at RT for 5 min before centrifuging (14k rpm x 5 min). The supernatant was removed, 500µL 70% EtOH added, and centrifuged (14k rpm x 3 min). The supernatant was again removed and the pellet allowed to dry (37°C x ~20 min) before resuspending in 20µL MQW. Approximately 20µg of DNA was

obtained using this method. Miniprep plasmid DNA was used for PCR, sequencing, restriction digestion, and some plasmid cloning.

2.2.9 Megaprep

Megaprep plasmid DNA was prepared from 500mL of overnight bacteria cultures, at 37°C with the antibiotic ampicillin (~50µg/mL) and was purified using the Jetstar 2.0 Mega plasmid purification kit (Astral) using the manufacturers protocol provided. DNA yield was determined by measuring a 1/100 dilution (in MQW) using UV spectrometry (260nm). Approximately 1-2mg of DNA was obtained using this method and stored in MQW at 4°C. Megaprep plasmid DNA was used for PCR, sequencing, restriction digestion, and plasmid cloning.

2.2.10 Subcloning of murine I-TAC

Sense and antisense oligonucleotide primers were designed to the target sequence. These oligonucleotides included EcoRI restriction sites to facilitate cloning. PCR amplification was used to amplify the target cDNA from a parental plasmid (section 2.2.5). The oligonucleotide primers used for this were, sense primer, 5'-TAGAATTCATGAACAGGAAGGTCACAGC-3' and antisense primer, 5'-TAGAATTCTCACATGTTTTGACGCCTTA-3'. The amplified fragment was cloned into the EcoRI site of pBluescript II KS$^-$ (Stratagene) to generate the new plasmid (2.2.7).

2.2.11 Baculovirus expression of murine I-TAC

A murine I-TAC recombinant baculovirus was generated using the Bac-to-Bac^{TM} Baculovirus expression system (PharMingen) using the supplied protocols. Briefly, the murine I-TAC ORF in pBSmI-TAC was excised and subcloned into the Xho1 and Xba1 restriction sites of the baculovirus vector plasmid, pFastBac1 (PharMingen). This plasmid was then transformed into competent DH10Bac *E. coli* cells that contain a wild type baculovirus genomic DNA bacmid. The resulting progeny were screened for recombinant bacmids, which were then used to transfect Sf9 insect cells (PharMingen). Sf9 cells were maintained in supplied media (Sf-900 II SFM, GibcoBRL) containing penicillin (50U/mL) and streptomycin (50µg/mL) at 27°C, 5% CO_2 according to suppliers protocol. On day 5, supernatant from Sf9 cells infected with either wild type

or recombinant baculovirus was harvested and concentrated 4 fold by centrifugation using the Centricon-3 microconcentrator (Amicon, USA) using the supplied protocol. An overview of the baculovirus expression system is illustrated.

Baculovirus expression system (PharMingen)

PFastBac donor plasmid

↓

Clone gene of interest

↓

PFastBac recombinant

↓

E. coli colonies with recombinant bacmid

↓

Verified *E. coli* colonies with recombinant bacmid

↓

Recombinant bacmid DNA

↓

Transfect insect cells

↓

Recombinant Baculovirus stock

↓

Protein expression

2.2.12 Splenocyte isolation and concanavalin A (Con A) blast induction

Whole spleens were isolated from euthanised mice and placed on wire mesh sieves over small culture dishes containing 2mL F15 MEM media (10% FCS, GibcoBRL). Splenocytes were isolated by gently homogenizing the spleen through the sieve using a 5mL syringe plunger. The splenocytes were then transferred into a 10mL tube and pelleted by centrifugation (1500 rpm for 5 min in a bench-top centrifuge). Supernatant was removed and cells suspended in 2mL RBC lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Disodium EDTA, pH 7.3, sterilized by filtration) and incubated for 2 min at RT. RBC lysis was stopped by adding 8mL F15 MEM (10% FCS), followed by two more washes. Splenocytes were resuspended at $5x10^6$ cells/mL in F15 MEM (10% FCS). Con A (Pharmacia Biotech) was added (2 µL of a 1mg/mL stock solution per mL cells) and the cells incubated at 37°C, 5% CO_2 for 24 or 48 hrs before use.

2.2.13 In vitro chemotaxis assay

Chemotaxis assays with Con A blasts were carried out using the Falcon Tissue culture insert system with 3 μm or 8 μm pore size 9 mm tissue culture inserts (Falcon), and companion tissue culture plates (Falcon), according to the manufacturers protocol. Briefly, the concentrated supernatant (section 2.2.11) was added (5-20μL) into F15 MEM (10% FCS, GibcoBRL) in the bottom of a 24-well chemotaxis plate. Washed Con A blasts ($4x10^5$ cells) were then added into the top of the tissue culture inserts while placing the inserts into the 24-well chemotaxis plate. After incubating at 37°C for 3 hrs the relative number of cells migrating to the lower surface was counted in five randomly chosen high-powered fields (magnification 100x) using a grid contained within a microscope eyepiece (Olympus). Multiple experiments were carried out to confirm the data.

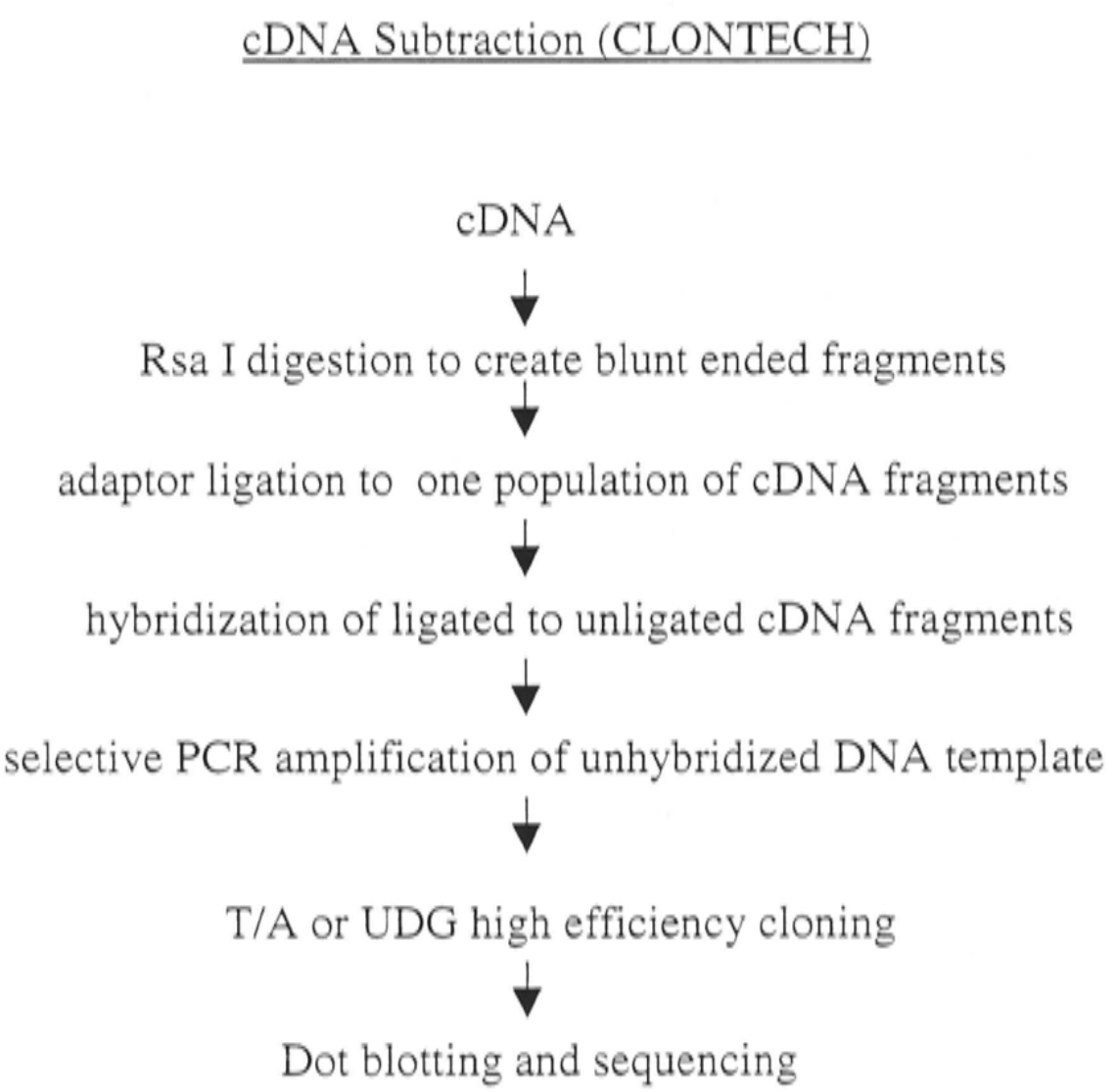
2.2.14 RNA isolation, cDNA synthesis and RT-PCR expression analysis in DC cell lines

Messenger RNA (mRNA) was isolated from the MTHC-D2 and JAWS II DC lines ($5x10^6$ cells) using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). First strand cDNA was prepared using a Ready-To-Go T-Primed First Strand Kit (Pharmacia Biotech) according to the manufacturer's instructions. Aliquots (1-2μL) of the first strand cDNAs were then used directly for RT-PCR (section 2.2.5).

2.3 Results

2.3.1 Identification of murine I-TAC

Previously, a subtraction cDNA library had been generated by subtracting the cDNA isolated from the parental murine MTHC-D2 DC line from cDNA isolated from the DCs after six hrs of stimulation with IFN-γ, GM-CSF and TNF-α (Banyer and Hapel, 1999). The subtraction technique follows the protocol in the flow diagram below.



A nucleotide blast search of partial sequences of a number of clones in this library identified the plasmid clone, pJB699, as having a cDNA fragment with significant homology to the cDNA for HuI-TAC. This murine cDNA sequence, termed murine I-TAC, was PCR amplified. Both PCR primers encoded EcoR1 sites enabling the recloning of murine I-TAC to generate the bluescript plasmid pBSmI-TAC (Fig 2.1).

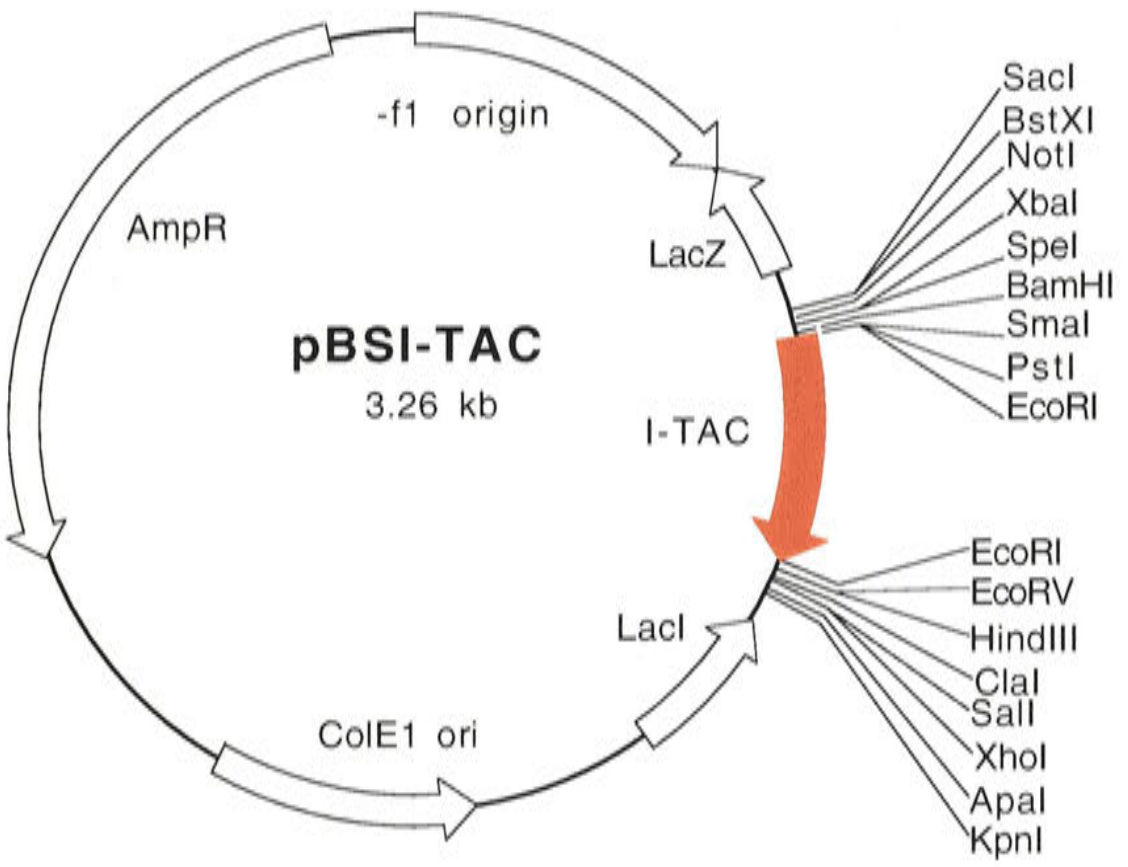


Figure 2.1: Plasmid map of Bluescript KS- encoding the complete I-TAC cDNA ORF. I-TAC was inserted into the EcoR1 restriction sites.

The cDNA of murine I-TAC in pBSmI-TAC was sequenced and confirmed with the sequence provided for pJB699. Locating the start codon (ATG) and stop codon (TGA) in this sequence and aligning the nucleotide sequence with HuI-TAC identified the ORF (Fig 2.2). By protein and nucleotide database homology analysis, it was demonstrated that the I-TAC protein is 100 amino acids long, six amino acids longer than HuI-TAC and is 67%, 37% and 32% homologous to HuI-TAC, Crg-2 (murine IP-10), and MuMig, respectively (Fig 2.3). Murine I-TAC, like these other chemokines, has the four conserved cysteine amino acid residues typical of CXC chemokines and does not contain an ELR motif (E-L-R refers to the amino acid motif required for recruiting neutrophils). Based on the hydrophobicity profile (Fig 2.4) of the ORF amino acid sequence and the comparisons with HuI-TAC, Crg-2 and MuMig, the signal peptide cleavage most likely occurs between Gly21 and Phe22 to produce a mature polypeptide of 79 amino acids long.

2.3.2 Chemotactic activity of I-TAC

Once the I-TAC open reading frame had been cloned and sequence analyzed, it was necessary to establish whether or not the murine I-TAC cDNA encoded a functional chemokine. This was carried out by construction of a recombinant baculovirus encoding the isolated cDNA by PCR reamplification and recloning I-TAC from pBSmI-TAC into the plasmid vector pFastBac1, using the Xba1 and Xho1 restriction enzyme sites (Fig 2.5). The pFastBacI-TAC vector was then transformed into competent DH10Bac *E. coli* cells containing wild type genomic baculovirus DNA (or bacmid), that combine to form recombinant progeny. A recombinant baculovirus encoding I-TAC and a control virus, were used to infect Sf9 insect cells. Six days later the supernatants were isolated and concentrated before being used in an *in vitro* chemotaxis assays with Con A activated splenocytes. A control virus was used to ensure that the virus did not induce cell migration. Supernatant containing I-TAC attracted more activated splenocytes than did supernatant produced using the control virus, which had levels comparable to media only samples. Figure 2.6 shows a representative of the experiments carried out with the 3 μm pore sized chambers. The 8 μm pore sized chambers had a higher background of splenocytes indicating that a number of cells were 'falling' through the pores rather than migrating through due to chemotaxis. At its highest level, I-TAC recruited at least 50% more activated splenocytes than both control samples. These findings confirmed that murine I-TAC had chemoattractant properties.

```
1/1                                                 31/11
atg aac agg aag gtc aca gcc ata gcc ctg gct gcg atc atc tgg gcc aca gct gct caa
M   N   R   K   V   T   A   I   A   L   A   A   I   I   W   A   T   A   A   Q

61/21                                               91/31
ggc ttc ctt atg ttc aaa cag ggg cgc tgt ctt tgc atc ggc ccc ggg atg aaa gcc gtc
G   F   L   M   F   K   Q   G   R   C   L   C   I   G   P   G   M   K   A   V

121/41                                              151/51
aaa atg gca gag gtc gag aaa gct tct gta att tac ccg agt aac ggc tgc gac aaa gtt
K   M   A   E   V   E   K   A   S   V   I   Y   P   S   N   G   C   D   K   V

181/61                                              211/71
gaa gtg att gtt act atg aag gct cat aaa cga caa agg tgc ctg gac ccc aga tcc aag
E   V   I   V   T   M   K   A   H   K   R   Q   R   C   L   D   P   R   S   K

241/81                                              271/91
cag gct cgc ctc ata atg cag gca ata gaa aaa aag aat ttt tta agg cgt caa aac atg
Q   A   R   L   I   M   Q   A   I   E   K   K   N   F   L   R   R   Q   N   M

301/101
tga
*
```

Figure 2.2: The cDNA and amino acid open reading frame sequence of the non-ELR CXC chemokine murine I-TAC is shown. Conserved cysteine residues are underlined.

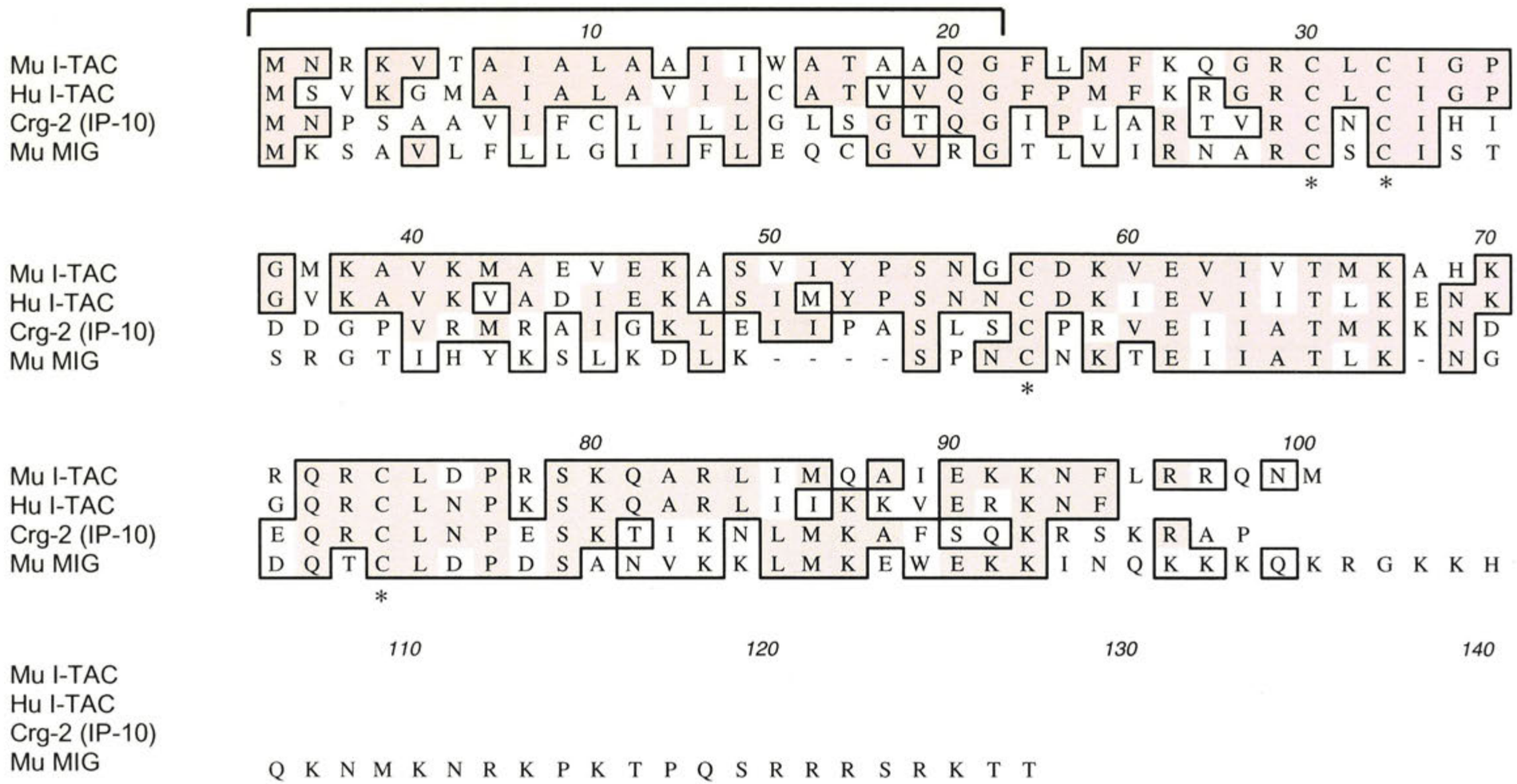


Figure 2.3: Sequence alignment. The sequence of murine I-TAC was aligned with three of the most homologous chemokines, human I-TAC, Crg-2 (murine IP-10) and murine MIG. The chemokine amino acid (ORF) sequences were aligned using a BLAST search and the ClustalW function of Mac Vector 6.0.1. Conserved regions between murine I-TAC and the other chemokines are shown within the shaded boxes. The proposed signal for murine I-TAC (cleaving between Gly21 and Phe22) is shown with a line. The conserved cysteine residues are indicated by an asterix below the sequences.

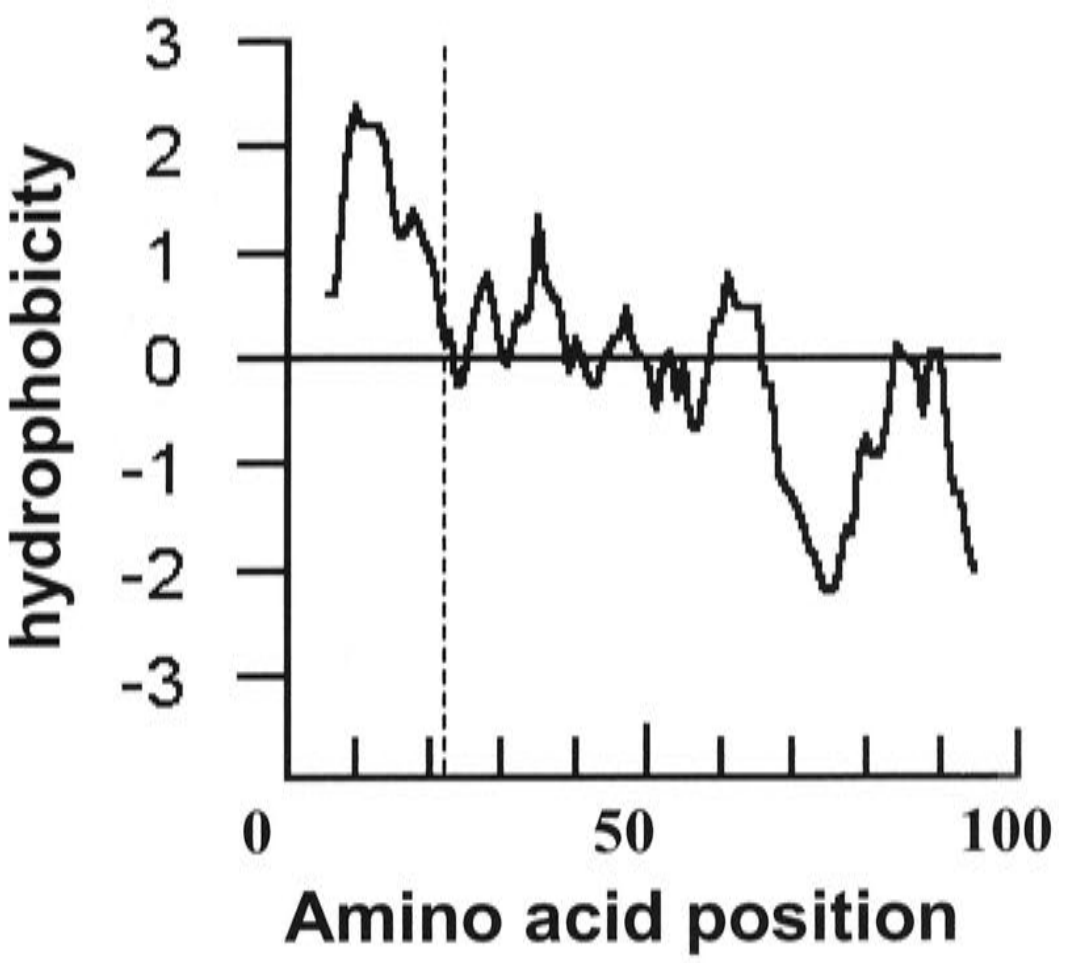


Figure 2.4: Kyte and Doolittle hydrophobicity plot of murine I-TAC generated using DNA-Strider software. The hydrophobic signal peptide is most likely cleaved between Gly21 and Phe22 as shown by the dashed vertical line.

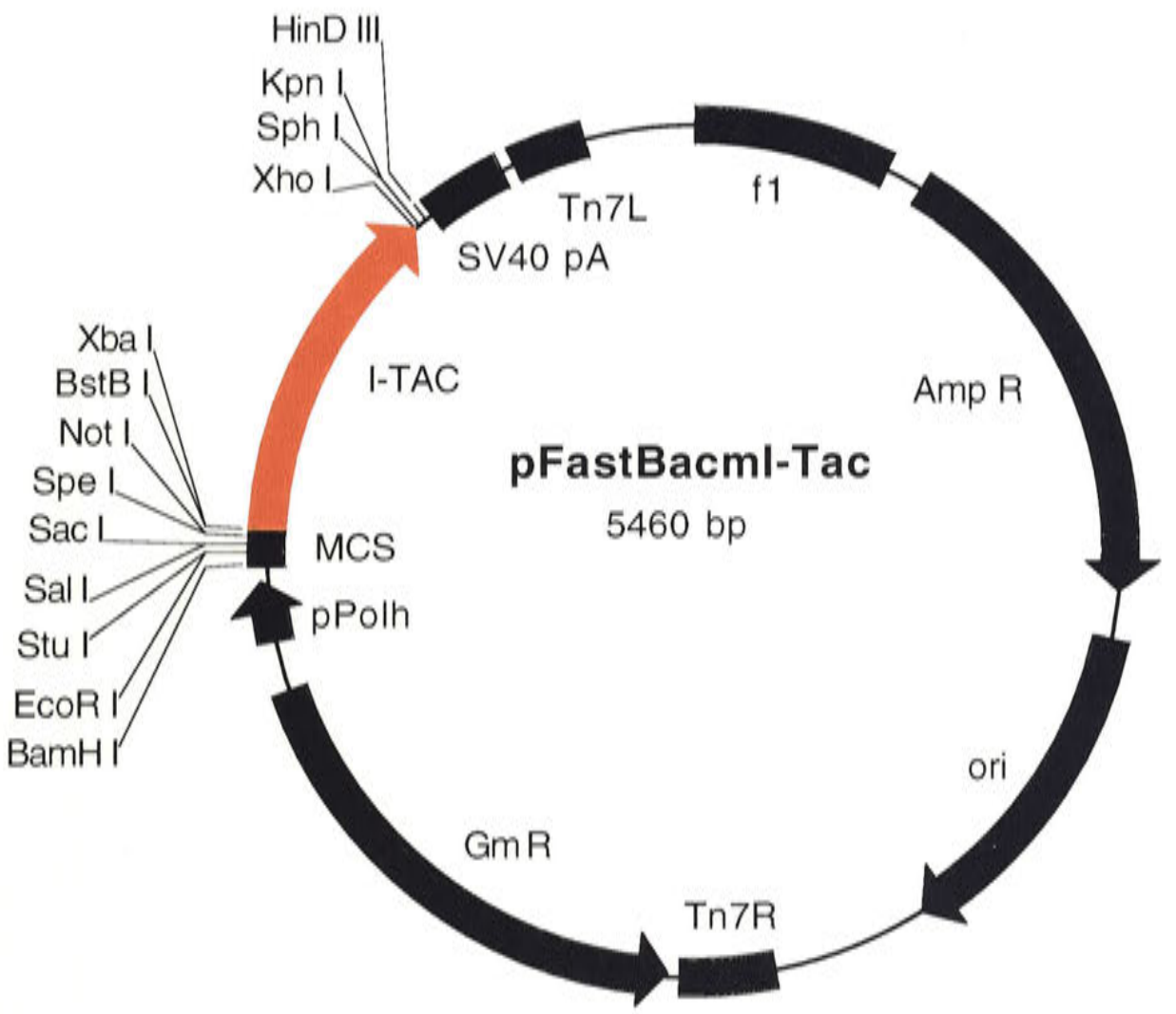


Figure 2.5: Plasmid map of pFastBac plasmid encoding the complete I-TAC cDNA ORF. I-TAC was inserted into the Xba1 and Xho1 restriction sites.

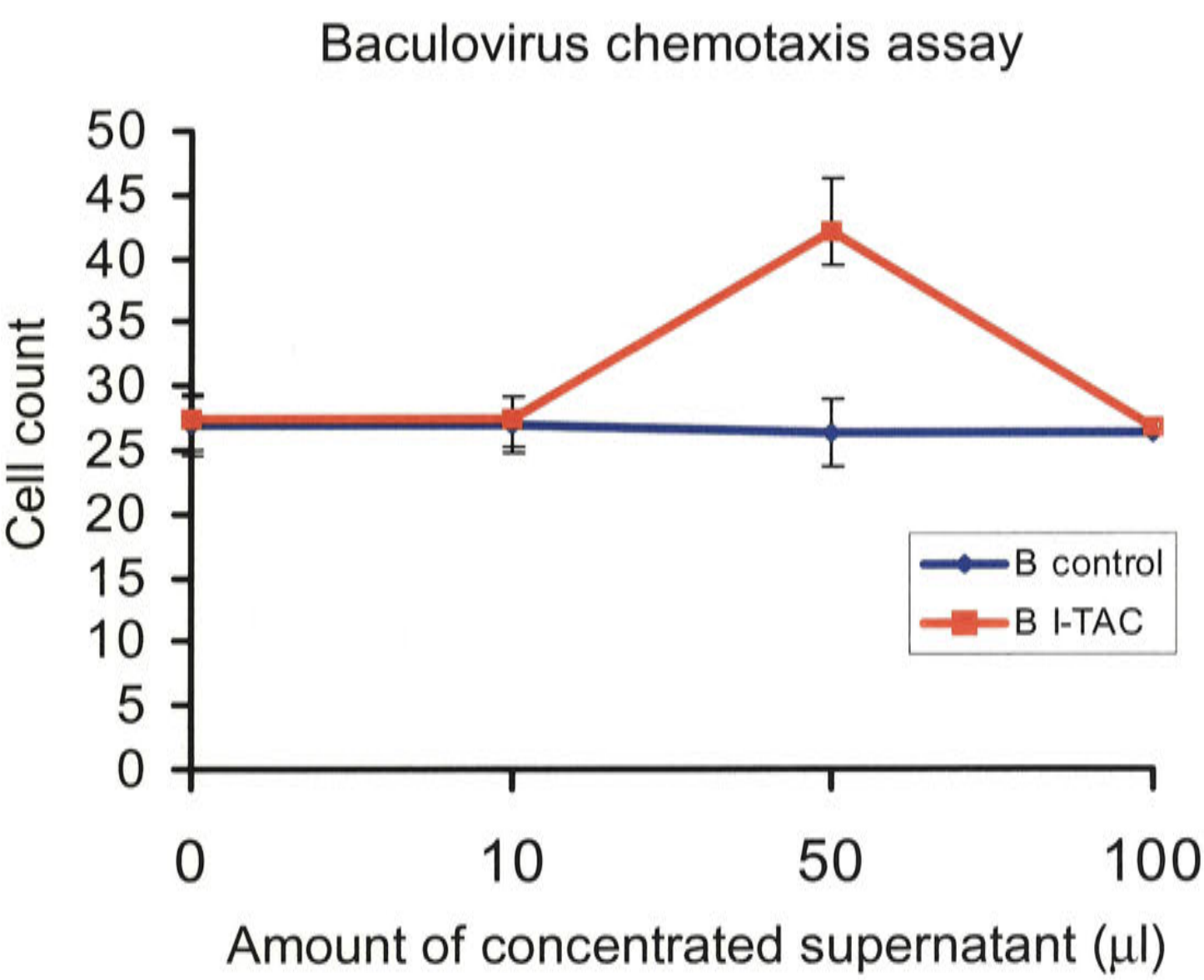


Figure 2.6: Baculovirus chemotaxis assay. Supernatant from a control virus or rB-I-TAC infected 143B cells was collected and concentrated ~4 fold and used for a chemotaxis assay. Con A blasted splenocytes (24 hrs stimulation) from naïve C57BL/6 mice were used for the assay. This figure is a representative of multiple experiments using the 3 µm pore sized chemotaxis chambers. Standard deviation is shown and there is a significant difference between rB-I-TAC and control virus at the 50 µl supernatant amount and media only samples (student's t-test $p<0.0002$).

2.3.3 RT-PCR from two DC lines

After finding that I-TAC was expressed from IFN-γ stimulated MTHC-D2 cells and could act as a chemoattractant molecule, a second DC cell line was examined for I-TAC expression. RT-PCR for murine I-TAC mRNA expression was carried out on the two DC cell lines, the MTHC-D2 cell line (Fig 2.7A) and the JAWS II cell line (Fig 2.7B). Both parental cells (lanes 1) had no detectable I-TAC whereas in IFN-γ stimulated cell samples (lanes 2), I-TAC was clearly visible. The housekeeping gene, GAPDH, was used as a positive control and for semi-quantification, demonstrated that the expression levels for I-TAC were similar in both DC lines.

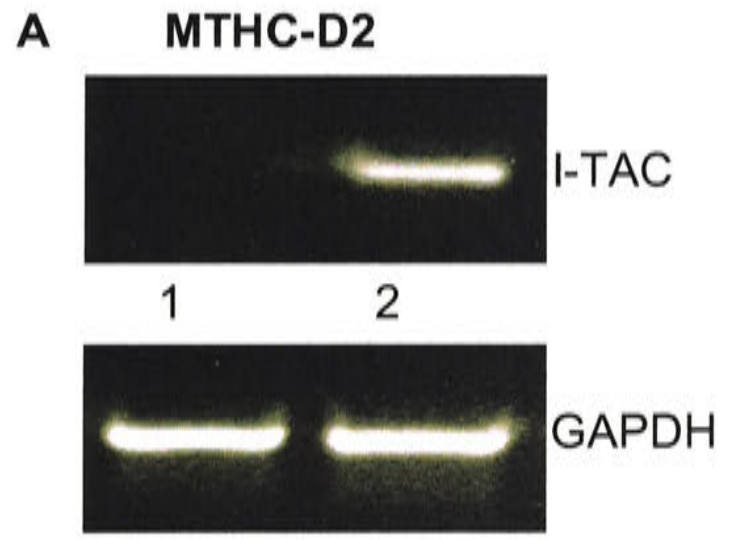


Figure 2.7: RT-PCR of I-TAC mRNA expression from the MTHC-2D cell line (A) and the JAWS II cell line (B). In both cell lines, I-TAC expression occurs after the parent cell line (lane 1) is stimulated with IFN-γ for 48 hrs (lane 2).

2.4 Discussion

The chemokine I-TAC was isolated and identified from the IFN-γ stimulated MTHC-D2 murine DC line following cDNA subtraction carried out by Dr Joanne Banyer (Banyer and Hapel, 1999). Murine I-TAC is 67% homologous to HuI-TAC and ~35% homologous to Crg-2 and MuMig respectively. There are four cysteine residues with the first two being separated by one amino acid, and as a result is a member of the non-ELR CXC chemokine family. The open reading frame is 100 amino acids long with a predicted 21 amino acid leader sequence. The predicted leader sequence coincides with the leader sequence for HuI-TAC and if cleaved at this point murine I-TAC would be a 79 amino acid residue mature protein.

A recombinant baculovirus encoding murine I-TAC demonstrated that the cloned cDNA encoded a protein that acts as a functional chemoattractant. A typical chemotactic response was observed with a reduction in the number of cells migrating at higher concentrations of I-TAC. This decrease in recruitment is a common chemokine regulatory mechanism caused by receptor desensitization (Ali *et al.*, 1999). HuI-TAC, HuMig and IP-10 as well as MuMig and Crg-2 are all induced by IFN-γ and attract activated T cells by binding to, and activating the human or mouse CXCR3 receptor respectively (Loetscher *et al.*, 1996; Cole *et al.*, 1998; Tamaru *et al.*, 1998; Lu *et al.*, 1999). Expression of murine CXCR3 is up regulated in Con A splenocytes (Tamaru *et al.*, 1998), and given the homology of the other CXCR3 binding chemokines to I-TAC, it is predicted that I-TAC also binds and activates the same murine receptor.

Previously it has been demonstrated that HuI-TAC is a more potent chemokine than either HuMig or IP-10 (Cole *et al.*, 1998). This was shown by several different methods, initially, intracellular calcium (Ca^{2+}) mobilization in activated T cells was measured. Dose response curves for HuI-TAC, IP-10 and HuMig on IL-2-stimulated T cell blasts showed that Ca^{2+} mobilization was highest for HuI-TAC, followed by IP-10, and then HuMig. The amount of Ca^{2+} mobilization was chemokine concentration dependent (Cole *et al.*, 1998). Following this, Cole *et al.*, further tested chemokine potency by looking at the interactions with CXCR3. Cross desensitization and binding of the three human chemokines to the CXCR3 receptor, again demonstrated that HuI-TAC was clearly the more potent chemokine of the three. Maximum chemotactic migration rates

of CXCR3 transfected cells were observed for HuI-TAC at a concentration of 10 nM versus approximately 100 nM required for both IP-10 and HuMig. In addition to this, twice as many stable transfectants migrated in response to HuI-TAC than towards the other two chemokines (Cole *et al.*, 1998). HuI-TAC was also able to displace IP-10 or HuMig binding to the CXCR3 receptor which again occurred in a concentration dependent manner (Cole *et al.*, 1998). Interestingly, neither HuMig nor IP-10 could completely displace HuI-TAC suggesting that HuI-TAC has either a much higher affinity for CXCR3 than the other two chemokines, or that it binds to the receptor at a different binding region (Cole *et al.*, 1998). The possibility of differential binding sites was investigated by Cox *et al.*, by carrying out competitive binding assays. Their research shows evidence that HuI-TAC binds to CXCR3 in a non-overlapping region to IP-10 and possibly HuMig, however HuI-TAC still remains the dominant ligand for the receptor (Cox *et al.*, 2001). The exact binding regions for each of these chemokines on the receptor are still unknown. Murine CXCR3 is highly homologous to the human CXCR3 receptor, with 86% identical amino acids (Lu *et al.*, 1999). While not examined here, the high homologies between the human and mouse chemokines make it plausible that murine I-TAC is also more potent than MuMig and Crg-2. It is therefore possible that murine I-TAC shares a lot of similarities to HuI-TAC with regard to binding affinities and potency in comparison to the Mig and Crg-2.

RT-PCR confirmed that murine I-TAC is expressed from murine progenitor DCs after cytokine stimulation. Together with the likelihood that murine I-TAC attracts T lymphocytes it is plausible that I-TAC maybe an important secondary messenger, linking the innate to adaptive immune systems. After DCs have been exposed to antigen and maturation signals (e.g. IFN-γ and TNF-α), a complete alteration in chemokine expression occurs. Expression of both Mig and IP-10 were also isolated from the MTHC-D2 cDNA library containing I-TAC, and have been reported by others to be upregulated during this time of DC maturation (Banyer *et al.*, 2002 submitted, see appendix 1c; Sallusto *et al.*, 1999; Sallusto and Lanzavecchia, 2000). The expression of these chemokines could have several functions including recruiting T lymphocytes to the DC for antigen presentation and sampling in the draining lymph nodes and subsequent adaptive immune response activation. Detection of these chemokines from the MTHC-D2 cells after only 6 hrs of cytokine stimulation suggests a role early in the

immune response, before the DC has migrated to the lymph node. Also, RT-PCR detection of I-TAC in MTHC-D2 cells after 48 hrs of cytokine stimulation suggests that this chemokine may still be functioning after the DC has reached lymph tissues (Banyer *et al.*, 2002 submitted, see appendix 1c). Another function could be to down regulate receptor expression and other factors no longer required by the mature DC such as those receptors previously required by the immature DC for migration to the site of the antigen (Sallusto and Lanzavecchia, 1999; Sallusto and Lanzavecchia, 2000). A more thorough examination of what cytokines and other stimulatory molecules regulate murine I-TAC may help determine what role this chemokine has in the immune system.

CHAPTER 3.

CHARACTERISATION OF MURINE I-TAC EXPRESSION IN CON A BLASTS OF KNOCKOUT MICE AND CYTOKINE MATURED CELL LINES

CHAPTER 3. CHARACTERISATION OF MURINE I-TAC EXPRESSION IN CON A BLASTS OF KNOCKOUT MICE AND CYTOKINE MATURED CELL LINES

3.1 Introduction

The expression of I-TAC from DC lines after stimulation with IFN-γ, GM-CSF and TNF-α suggests that I-TAC may be involved with CMI responses. Characterisation of the signalling pathway(s) responsible for the expression of I-TAC will help further define the role it has in the immune system.

3.1.1 The interferon (IFN) signalling pathways

The IFN regulatory pathway is made up of multiple cytokines, receptors and transcription factors, which together can effect the expression of a number of chemokines (Fig 3.1) (reviewed in Stark *et al.*, 1998; Platanias and Fish, 1999; Schindler, 1999). There are two main IFN receptors, the type I receptor (IFN-α/βR) and type II receptor (IFN-γR) which signal through related pathways. Both receptors signal through the binding to the Janus kinases (JAKs) which initiate signals through the signal transducers and activators of transcription (STATs). These JAK and STAT factors are not exclusive to IFN signalling but are also involved in other cytokine and growth factor signalling pathways. There are four known mammalian JAKs and seven known STAT factors (reviewed in Stark *et al.*, 1998; Platanias and Fish, 1999; Schindler, 1999).

Once the IFN-α/βR binds ligand it activates two JAKs, JAK1 and Tyk2 which in turn, activate STAT1 and STAT2 by a tyrosine phosphorylation event. These two STATs migrate to the nucleus where they bind with p48 (also known as Interferon response factor 9 – IRF9) to form the IFN-α-stimulated gene response factor (ISGF) complex. ISGF then binds to the IFN-α-stimulated response element (ISRE) to then mediate the induction of target genes (reviewed in Stark *et al.*, 1998; Platanias and Fish, 1999; Schindler, 1999).

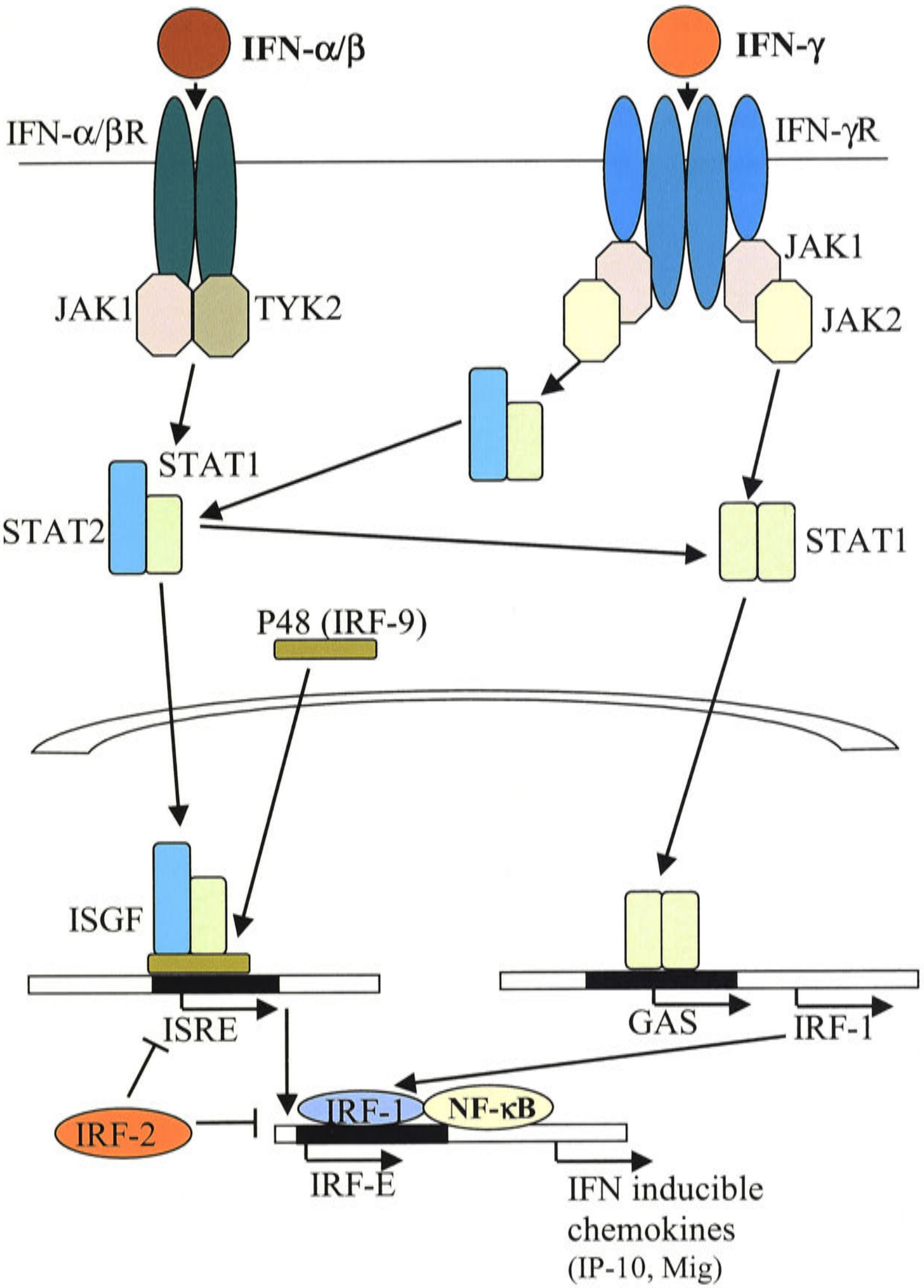


Figure 3.1: Schematic diagram of the IFN signalling pathway through the STAT-JAK factors (adapted from Stark *et al.*, 1998; Schindler, 1999; Taniguchi *et al.*, 2001).

IFN-γ has a similar sequence of events, however, the proteins which are activated differ to some extent. Once the IFN-γR has been activated, JAK1 and JAK2 are activated and initiate tyrosine phosphorylation of two STAT1 proteins. The activated STAT1 factors form a homodimer that migrates to the nucleus to bind to the gamma activation site (GAS), which is an IFN-γ response element that initiates the induction of target genes. The target genes include the interferon response factor-1 (IRF-1) leading to expression of antiviral factors such as the IFN-γ inducible chemokines, IP-10 and Mig (reviewed in Stark *et al.*, 1998; Platanias and Fish, 1999; Schindler, 1999). IRF-1 is a major factor in NK cell antiviral activity as well as in the development of Th1 cell differentiation. IRF-1 physically interacts with the transcription factor NF-κB and it has been suggested that this binding may be important in the cytokine-induced regulation of multiple promoters (Baldwin, 1996). The antiviral activity of IRF-1 and subsequently NF-κB, is down regulated by IRF-2 through suppression of ISRE and IRF-E (Fig 3.1), (Taniguchi *et al.*, 2001).

3.1.2 TNF-α signalling

Studies examining TNF signallinghavebeen driven by the knowledge that this cytokine family induces apoptosis as well having a major role in cell growth and homeostasis (reviewed in Lenardo *et al.*, 1999; Wallach *et al.*, 1999). Although these effects seem contradictory, the mechanism by which it operates ensures the survival of healthy, stable cells. TNF-α mediates its cellular responses through two distinct receptors, TNFR-I (p55) and TNFR-II (p75). These receptors are then able to activate numerous other factors, for example TNFR-I can associate with TRADD, FADD, TRAF2, I-TRAF, RIP and FAN while TNFR-II associates with factors including TRAF1, TRAF2, cIAP-1 and cIAP-2 (reviewed in Darnay and Aggarwal, 1997). The TNFR-I activation of TRADD leads to the activation of TRAF2, which can also be activated by TNFR-II directly. TRAF2 activation subsequently leads to the activation of several factors including A-20 and I-TRAF which in turn have the ability to activate NF-κB (Rothe *et al.*, 1995; Hsu *et al.*, 1996). Synergy between TNF-α and IFN-γ to induce chemokine expression, including HuI-TAC and IP-10 has been reported (Ohmori and Hamilton, 1995; Gasperini *et al.*, 1999; Sauty *et al.*, 1999; Albanesi *et al.*, 2000). The signalling mechanism for this synergistic effect is not entirely clear, however, both IFN-γ and

TNF-α can independently activate ISRE and NF-κB, both of which are involved in chemokine induction, including that of IP-10 (Ohmori and Hamilton, 1995). A schematic diagram can be seen on the previous page (Fig 3.1a)

3.1.2 CD40-CD40L signalling

Interactions between cytokines and co-stimulatory molecules can also regulate chemokine expression. The cell surface receptor CD40 belongs to the TNF-receptor (TNF-R) family and was first identified on B-lymphocytes, and has since been found to be expressed on monocytes, DCs and a number of nonhemopoietic cells including keratinocytes (reviewed in Lipsky *et al.*, 1997; van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). The major role of CD40 is in the interaction with its ligand, CD40L, which is expressed on activated $CD4^+$ T cells and as a result functions in B cell differentiation and activation during humoral immune responses (reviewed in Lipsky *et al.*, 1997; van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). Functional CD40 expression on DCs also appears to be critical in DC maturation as well as having a role in inflammatory immune responses (Mackey *et al.*, 1998). Upon DC activation via CD40 a number of chemokines and cytokines are produced including IL-8, MIP-1α, IL-12, IL-1 and TNF-α. The DC CD40 induced expression of factors such as IL-12 and subsequent interaction and activation with T cells results in a skewing towards a Th1 response (van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). In addition to this, synergy between CD40-CD40L interactions and IFN-γ to induce expression of several chemokines in cervical carcinoma cells has been observed. The chemokines upregulated include MCP-1, IP-10, and to a lesser extent MCP-3 and RANTES (Altenburg *et al.*, 1999).

The CD40 signalling pathway has been primarily studied in B cells although there maybe alternative pathways functioning in other cells such as in DCs (van Kooten and Banchereau, 1997). The intricacies of CD40 signalling are still unclear however several features are known. Similar to all other members of the TNF receptor family, CD40 has no kinase binding or activation domain, yet CD40 ligation activates protein tyrosine kinase including Syk, Lyn and JAK3 kinases. Several MAP kinases also appear to be activated by CD40 ligation. A number of TNF-R associated factors (TRAFs) have also found to be involved in the CD40 signalling pathway. The TRAFS can then induce several transcription factors, for example TRAF2 can induce NF-κB following CD40

cross-linking (van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). Another factor that interacts with CD40 is the tumour-suppressor gene, p53, which is involved in cell cycle regulation. Indeed, activation of multiple myeloma cells by CD40 induces proliferation, growth arrest or apoptosis, all of which are dependent on the status of p53 (Teoh *et al.*, 2000).

Establishing how immunologically important factors including IFN-γ, TNF-α and CD40 ligand can influence murine I-TAC expression will help establish the role and importance of murine I-TAC during immune responses.

3.2 Materials and Methods

3.2.1 Mice

Specific pathogen-free 6-8 week old female mice were obtained from the Animal Breeding Facility, John Curtin School of Medical Research, Canberra, Australia. Single knockout IFN-$\alpha/\beta R^{-/-}$ and IFN-$\gamma R^{-/-}$ mice and IFN-α/βR-$\gamma R^{-/-}$ double knockout mice homozygous for the disrupted gene(s) and backcrossed onto the 129/Sv background, were originally obtained from Dr Michel Aguet, University of Zurich (Huang *et al.*, 1993; Steinhoff *et al.*, 1995; van den Broek *et al.*, 1995). Interferon regulatory factor-1 KO (IRF-$1^{-/-}$) mice backcrossed onto the 129/Sv background were originally obtained from Prof. Charles Weissman, University of Zurich (Reis *et al.*, 1994). $CD40^{-/-}$ mice were obtained from Prof. Hitoshi Kikutani, Osaka University (Kawabe *et al.*, 1994). TNF-IR-$IIR^{-/-}$ double knockout mice were obtained from Dr Jacques Peschon, Immunex Research and Development Corporation (Peschon *et al.*, 1998). Two sources of age-matched null homozygote (C57BL/6 x 129/Sv) mice were used as controls for these assays (Max-Planck Institute for Immunbiologie, Freiburg, Germany and University of Zurich, Switzerland) as described previously (Ramshaw *et al.*, 1997; Ruby *et al.*, 1997).

3.2.2 Semi-quantitative RT-PCR of DC lines exposed to cytokines and CD40 co-stimulation

JAWS II and MTHC-D2 cell lines were cultured as described in section 2.2.2. In addition, they were stimulated for 48 hrs with TNF-α (500 U/mL) and GM-CSF (5u/mL) and either IFN-γ (2U/mL) or IL-4 (50U/mL), or both IFN-γ plus IL-4. Some cell samples were activated for the final 16 hrs by CD40 crosslinking by adding CD40 Ab (25μg/mL). The CD40 Ab was column-purified from supernatant of cultured FGK45.5 cells (Rolink *et al.*, 1996). Semi-quantitative RT-PCR was carried out after mRNA isolation (from $1x10^6$ cells) and cDNA synthesis as described in sections 2.2.5, 2.2.12 and 2.2.14. These analyses were carried out multiple times to confirm the data.

3.2.3 Semi-quantitative RT-PCR of Con A blasts from knockout mice

Semi-quantitative RT-PCR was carried out after mRNA isolation (from $1x10^6$ cells) and cDNA synthesis as described in sections 2.2.5, 2.2.12 and 2.2.14. Multiple mice were used and RT-PCRs repeated at least three times to confirm the data.

3.3 Results

3.3.1 Cytokine induced expression of murine I-TAC in DC lines

I-TAC expression was induced in DCs matured by IFN-γ and TNF-α (chapter 2). A common approach used to culture and mature DCs is with IL-4 (Sallusto and Lanzavecchia, 1994; Banyer and Hapel, 1999). To investigate what role IL-4 may have on I-TAC expression, RT-PCR analysis of the MTHC-D2 and JAWS II cell lines exposed to TNF-α plus IL-4, or TNF-α plus IFN-γ cytokines was carried out. These two cell lines are progenitor cells of DC isolated from different tissues and which have been transformed by two different approaches. Semi-quantitative RT-PCR analysis of both the MTHC-D2 (Fig 3.2A) and JAWS II (Fig 3.2B) cell lines showed that expression of murine I-TAC was upregulated by IFN-γ/TNF-α (lanes 3) compared to the IL-4/TNF-α (lanes 2) or untreated cells (lanes 1) both of which had no detectable murine I-TAC band. These data also indicate indirectly that IL-4 and TNF-α alone do not upregulate I-TAC expression and that the key cytokine for expression appears to be IFN-γ. Indeed when IL-4/IFN-γ/TNF-α (lanes 7) were combined I-TAC expression was significantly lower than IFN-γ/TNF-α (lanes 3) treated cells indicating that IL-4 may in fact down-regulate IFN-γ induced I-TAC expression.

3.3.2 CD40 Ab induced expression of murine I-TAC in DC lines

Synergy between IFN-γ and CD40 has been shown to enhance the expression of a number of chemokines from cervical carcinoma cells, including IP-10 and MCP-1 (Altenburg *et al.*, 1999). Considering the high homology between murine I-TAC and IP-10 (chapter 2) and their induction by IFN-γ, it was important to examine the effects of CD40 on murine I-TAC expression. Semi-quantitative RT-PCR analysis, standardized against GAPDH, showed, that in the absence of IFN-γ, anti-CD40 Ab upregulated the expression of murine I-TAC in MTHC-D2 cells (Fig 3.2A lanes 1 and 2 vs lanes 4 and 5 respectively), but not in the JAWS II cell line (Fig 3.2B lanes 1 and 2 vs lanes 4 and 5 respectively). I-TAC expression was upregulated further in the MTHC-D2 cell line when exposed to both CD40Ab and IFN-γ demonstrating synergy (Fig 3.2A lane 3 vs lane 6) whereas synergy in the JAWS II was not detected (Fig 3.2B lane 3 vs lane 6). The different I-TAC expression pattern between the cell lines may be due to differences in the CD40 activation pathway resulting from the different methods used to immortalize the cell lines, rather than due to interactions with the interferon-signalling

pathway. A combination of IL-4/IFN-γ/TNF-α plus anti-CD40 Ab (lanes 8) appears to down-regulate I-TAC expression compared to IFN-γ/TNF-α plus anti-CD40 Ab (lanes 6) treated cells, again indicating that IL-4 down-regulated IFN-γ/TNF-α/CD40 induced I-TAC expression.

3.3.3 Expression of murine I-TAC Con A blasts from knockout mice

To further analyze murine I-TAC expression and confirm the cell line results, semi-quantitative RT-PCR, standardized against GAPDH, was carried out on cDNA from non-activated and Con A activated splenocytes isolated from a panel of knockout mice. Murine I-TAC expression in non-activated splenocyte cDNA samples from the various transgenic mice and controls was very low and not significantly different between strains of mice (data not shown). In Con A activated splenocyte samples (Fig 3.3) there was a significant increase in the level of murine I-TAC expression in splenocytes from IFN-α/βR$^{-/-}$ (lane 3) mice compared to control spleen cells (lanes 1 and 2). These results appeared to indicate that IFN-α and/or IFN-β down regulate murine I-TAC expression in wild type mice. Expression of murine I-TAC in splenocytes from IFN-γR$^{-/-}$ (lanes 4), IFN-α/βR-γR$^{-/-}$ (lanes 5) and IRF-1$^{-/-}$ (lanes 6) mice were all significantly lower than the expression in the splenocytes from control mice (lanes 1 and 2). These results showed that the IFN-γ signalling pathway is the primary regulator of murine I-TAC mRNA expression and that IRF-1 has a significant role in murine I-TAC expression supporting the cell line data above. Murine I-TAC expression in activated splenocytes from CD40$^{-/-}$ (lane 7) mice was not significantly different to the samples from control mice (lanes 1 and 2). This corresponds to the *in vitro* data for the JAWS II cell line (Fig 3.2B) but not to the data for the MTHC-D2 cell line (Fig 3.2A). There was no significant difference between the level of murine I-TAC detected in TNF-IR-IIR$^{-/-}$ (lane 8) mice compared to control mice (lanes 1 and 2). This suggests that TNF-α does not have an important role in regulating murine I-TAC mRNA, in agreement with the *in vitro* cell line data.

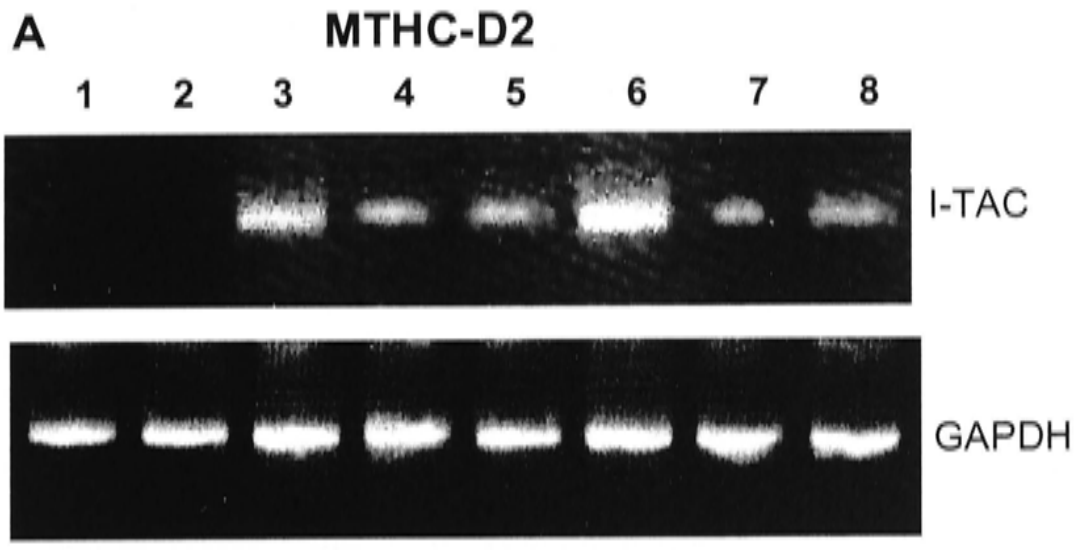


Figure 3.2: Semi-quantitative RT-PCR murine I-TAC and GAPDH mRNA expression in the MTHC-D2 (A) and JAWS II (B) cell lines grown in GM-CSF only (lane 1) and following exposure to the cytokines; IL-4/TNF-α (lane 2), IFN-γ/TNF-α (lane 3), GM-CSF plus anti-CD40 Ab (lane 4), IL-4/TNF-α plus anti-CD40 Ab (lane 5), IFN-γ/TNF-α plus anti-CD40 Ab (lane 6), IL-4/IFN-γ/TNF-α (lane 7), and IL-4/IFN-γ/TNF-α plus anti-CD40 Ab (lane 8).

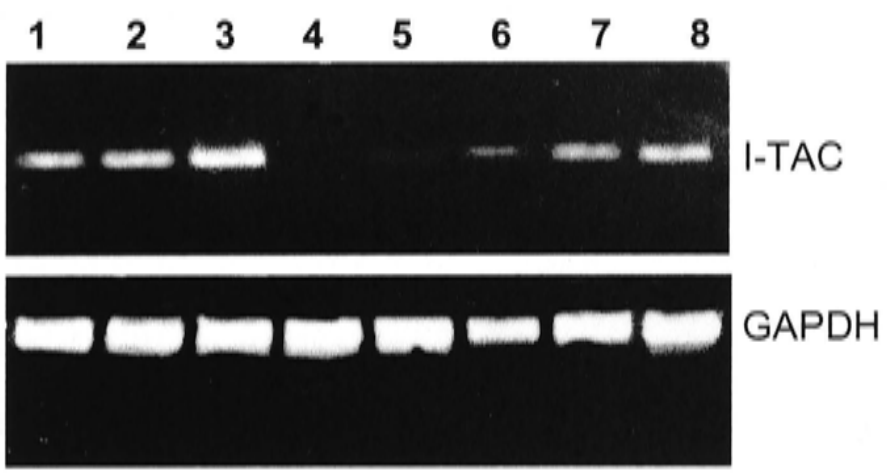


Figure 3.3: Murine I-TAC and GAPDH semi-quantitative RT-PCR analysis of Con A activated splenocytes from a panel of knockout mice. Two sources of age-matched littermate null homozygote (129/Sv x C57BL/6) mice were used as controls (lanes 1 and 2). The panel of knockout mice were as follows: IFN-$\alpha/\beta R^{-/-}$ (lane 3), IFN-$\gamma R^{-/-}$ (lane 4), and IFN-α/βR-$\gamma R^{-/-}$ (lane 5), IRF-$1^{-/-}$ (lane 6), $CD40^{-/-}$ (lane 7), and TNF-IR-$IIR^{-/-}$ (lane 8). The cDNA samples from three individual mice in each case were obtained then combined equally prior to PCR amplification for this figure. Individual samples were analysed multiple times to ensure reproducibility.

3.4 Discussion

Previous studies suggest that APCs are an important source of I-TAC (Cole *et al.*, 1998). Expression of I-TAC from IFN-γ activated DCs was shown in chapter 2. The more detailed analysis here confirmed that murine I-TAC expression from DCs is regulated primarily by the IFN-γ signalling pathway and appears to be similar to the regulation of HuI-TAC, IP-10 and Mig expression. A significant finding was that IL-4, a cytokine frequently used to stimulate ex-vivo DCs, does not induce I-TAC expression and in fact appeared to decrease IFN-γ induced I-TAC expression. Both the Th1 cytokines, such as IL-12 and IFN-γ, and Th2 cytokines including IL-4, can suppress each other. This negative regulation appears to occur at several different levels during cytokine signalling. (reviewed in Rook *et al.*, 1994; Ivashkiv, 1995; Yasukawa *et al.*, 2000). Regulation of STAT activity may occur by inhibition or activation of the STATs themselves, upstream signalling events and also at the DNA promoter level (reviewed in Ivashkiv, 1995). For example, IL-4 activates the STAT6 transcription factor, which can bind to the same promoter, FcγR1, as the IFN-γ activated STAT1 factor leading to competitive regulation (Larner *et al.*, 1993). One of the ways IL-4 could antagonize IFN-γ action and subsequently CMI responses may include the down regulation of I-TAC via the inhibition of the IFN-γ signalling pathway.

Regulation of murine I-TAC expression by cytokines and downstream signalling factors was further examined in Con A activated splenocytes from a panel of knockout mice. Expression of murine I-TAC in splenocytes from IFN-$\gamma R^{-/-}$ and IFN-α/βR-$\gamma R^{-/-}$ mice were barely detectable compared to I-TAC expression in the splenocytes from control mice which confirms the cell line data. Together, these results confirmed that IFN-γ is the primary regulator of murine I-TAC. Mice lacking IFN-γR appear to develop a normal immune system, however they are far more susceptible to infection by *Listeria monocytogenes* and VV despite apparently normal cytotoxic and T helper cell responses (Huang *et al.*, 1993). Although IFN-γ initiates a number of anti-pathogen mechanisms, it is likely that significantly reduced levels of I-TAC and probably IP-10 and Mig in these mice, may reduce migration of immune cells to the site of infection and thereby increasing the susceptibility of these mice to infection. In a similar fashion, IFN-α/βR-$\gamma R^{-/-}$ mice are also susceptible to lymphocyte choriomeningitis virus (LCMV) and VV (van den Broek *et al.*, 1995). The replication rate of VV is much higher in these mice

and there was a deficient CTL response against the virus (van den Broek *et al.*, 1995). Again, it is likely that in these mice, a lack of I-TAC may decrease the migration of T lymphocytes to the infection site, reducing the ability of these mice to mount an effective immune response.

The assays carried out here, also demonstrated that a proportion of I-TAC expression is dependent on the action of IRF-1, with the remainder of I-TAC expression possibly being regulated by other IRF transcription factors involved in the interferon signalling pathway (reviewed in Taniguchi *et al.*, 2001). The finding that murine I-TAC is in part regulated by IRF-1 may help explain some of the complex phenotype of IRF-$1^{-/-}$ mice. IRF-$1^{-/-}$ mice have defective development of thymic $CD8^+$ cells, severe NK cell deficiency and an abnormally high proportion of Th2 $CD4^+$ T cells, which together lead to increased susceptibility to pathogens normally controlled by Th1 responses (Mamane *et al.*, 1999). Thus the decreased expression of murine I-TAC from DCs in IRF-$1^{-/-}$ mice, combined with decreased Crg-2 and MIG expression (S Mahalingam, unpublished data), may be altering the balance of Th1/Th2 $CD4^+$ T cells and subsequent susceptibility to infection.

Previously it has been shown that IFN-α or IFN-β can enhance the expression of HuI-TAC with or without other cell activation signals (Rani *et al.*, 1996). In this study however, using the cells from various knockout mice, these two cytokines instead, appeared to down regulate the level of murine I-TAC expression. The level of these cytokines in the cultures while not analyzed, was probably low (Watanabe and Kawade, 1988; Noronha *et al.*, 1993). These analyses and others have shown that low levels of IFN-α or IFN-β can increase the expression of IRF-2, which competes with IRF-1 thereby suppressing IRF-1-regulated genes (Mamane *et al.*, 1999). Therefore in these assays, the lack of IFN-α and/or IFN-β signalling in the IFN-α/β$R^{-/-}$ and IFN-α/β-γ$R^{-/-}$ Con A cultures, and subsequent lower levels of IRF-2 expression, explains why murine I-TAC expression was relatively higher than in the wild type and IFN-γ$R^{-/-}$ Con A cultures respectively. Thus, comparing this data with previously published human I-TAC findings, it appears that IFN-α and IFN-β can act both to increase or decrease I-TAC expression depending on the relative amount of these two cytokines present (Huang *et al.*, 2000).

Others have reported that TNF-α and IFN-γ synergize to enhance the expression of HuI-TAC (Gasperini *et al.*, 1999; Sauty *et al.*, 1999; Albanesi *et al.*, 2000). No evidence of synergy between IFN-γ and TNF-α in either the cell line or Con A culture analyses was found in these studies. In these cell line assays TNF-α is included primarily to promote cell differentiation during stimulation with other cytokines (Banyer and Hapel, 1999). Hence, in the analyses reported by others, TNF-α may have appeared to enhance expression of I-TAC induced by IFN-γ through increased survival of differentiated cells.

IFN-γ, TNF-α and CD40 have all been shown to activate the NF-κB transcription factor, therefore it is of no surprise then, that a synergistic effect is observed between IFN-γ and either TNF-α or CD40 to induce IP-10 and Mig (Ohmori and Hamilton, 1995; Baldwin, 1996; van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). However, the synergistic induction of IP-10 by IFN-γ and TNF-α occurs via activation of NF-κB through independent binding sites (Ohmori and Hamilton, 1995). This effect can also be cell type and/or stimulus specific. For example, this cooperative effect between TNF-α and IFN-γ to induce IP-10 is observed in NIH 3T3 cells, whereas in macrophages, no synergy is detected (Ohmori and Hamilton, 1995; Huang *et al.*, 2000). Therefore, another explanation for the variability in observing synergy between these cytokines in the induction of murine I-TAC may be that NF-κB activation in the DC cell lines in this study was different to NF-κB activation in the cells used in previous reports for IP-10 and Mig.

The addition of anti-CD40 Ab to MTHC-D2 cells showed that CD40 alone can upregulate expression of murine I-TAC and that the CD40 and IFN-γ pathways can synergize to enhance this expression. This is consistent with a report by Altenburg *et al*, who showed that in cervical carcinoma cells, IFN-γ can synergize with CD40L to enhance expression of a number of chemokines including IP-10, MCP-1, and to a lesser extent RANTES and MCP-3 (Altenburg *et al.*, 1999). Enhanced I-TAC expression however, was not detected in the JAWS II cell line following CD40 activation. This result could be due to differences in the level of CD40 expression between the two cell lines. The JAWS II cell line however, lacks the tumor suppressor p53 and others have shown that p53-dependent functions can be regulated by CD40 (Teoh *et al.*, 2000).

Hence, the absence of IFN-γ/CD40 synergy in the JAWS II cell line could be explained by poor responsiveness in some of the downstream CD40 signalling pathways which involve p53. An alternative explanation is that the CD40 signalling pathways in the MTHC-D2 line was somehow enhanced due to the unregulated expression of truncated c-Myb used to immortalize these cells (Gonda *et al.*, 1993). In the Con A activated $CD40^{-/-}$ splenocyte cultures, the lack of CD40 did not appear to reduce murine I-TAC expression, which like the JAWS II cell line suggests that CD40/IFN-γ do not synergize to enhance I-TAC expression. In addition to this the effect of TNF-α and/or CD40 could be obscured by large amounts of IFN-γ in the Con A activated splenocyte cultures. Therefore, Con A may not be an appropriate stimulus to observe synergy between these cytokines.

In summary, these data indicated I-TAC is primarily induced by IFN-γ through the transcription factor IRF-1. CD40 may have a synergistic effect on expression, probably also via the activity of IRF-1. Together, with the data from chapter 2, I-TAC seems to be expressed from maturing DCs. If murine I-TAC is like human I-TAC, then it too probably enhances Th1 immune responses by attracting activated $CD4^+$ T lymphocytes to the site of infection. Examining different disease models would clarify whether or not this chemokine has a functional role in immune defense.

CHAPTER 4.

I-TAC EXPRESSION DURING DISEASE

CHAPTER 4. I-TAC EXPRESSION DURING DISEASE

4.1 Introduction

There are many disease models where I-TAC may have a role, especially in diseases where the CMI response is activated. To examine this, the expression of I-TAC in respiratory syncytial virus (RSV), influenza virus and vaccinia virus (VV) infection models as well as the auto immune disease model experimental autoimmune encephalomyelitis (EAE) and non-rejected allo- and xenograft transplantation models were investigated.

4.1.1 Chemokine expression initiated by viral infections

Virus infection leads to the release of a myriad of chemokines that activate the immune system. Indeed, many chemokines have been detected during viral infections, for example expression of RANTES, MCP-1, MIP-1α and IP-10 has been detected during an influenza A virus infection of human monocytes (Bussfeld *et al.*, 1998; Matikainen *et al.*, 2000). Influenza A primarily infects epithelial cells in the upper respiratory tract, as well as infecting macrophages, monocytes and T cells. After infection, a number of different cells migrate to the lungs and respiratory tract in response to the multiple chemokines released (Bussfeld *et al.*, 1998; Matikainen *et al.*, 2000).

A large number of chemokines are also expressed by respiratory syncytial virus (RSV) infected human lower airway epithelial cell lines (A549 and SAE cells) including, MIP-1α, MIP-1β, RANTES, GRO-α, GRO-β, GRO-γ, IP-10 and HuI-TAC (Zhang *et al.*, 2001). RSV causes a lower respiratory tract disease in young children and is associated with obstruction of the airways and enhanced inflammation and infiltration of a number of different leukocytes (Chanock *et al.*, 1992). The expression of RANTES induced during RSV infection is dependent on the transcription factor NF-κB. Indeed RSV has been shown to induce NF-κB, which is also important for IP-10 and HuI-TAC expression (Thomas *et al.*, 1998; Roebuck *et al.*, 1999; Zhang *et al.*, 2001).

The IFN-inducible chemokines Mig and IP-10 have been shown to be upregulated during several viral infections, although expression may be dependent on either virus strain or host strain. Ectromelia virus (EV), an orthopoxvirus closely related to VV, is a

natural mouse pathogen that causes generalized infection resulting in a disease termed mousepox. Inbred strains of mice may be classified as resistant or susceptible to mousepox. For instance, C57BL/6 mice are resistant to the disease while BALB/c mice are highly susceptible. These differences are attributed to the generation of a Th1 immune response in resistant mice, and a Th2 immune response in susceptible mice (Mahalingam *et al.*, 2001). It has been shown that the mRNA and protein expression of Mig and Crg-2 are up-regulated in the draining lymph nodes of resistant C57BL/6 mice but not in those of susceptible BALB/c mice as early as one day after infection with EV (Mahalingam *et al.*, 2000). Local treatment of BALB/c mice with recombinant Crg-2 shortly after infection, however, results in enhanced NK cell activity in the draining lymph node. The data suggest that the lack of NK cell-activating chemokines contribute to the suboptimal NK cell-mediated defense. Therefore, the early production of Mig and Crg-2 may also contribute to the resistance to EV by affecting the circulating population of activated T cells (Mahalingam *et al.*, 2000).

During VV infections, numerous chemokines are expressed including Mig and Crg-2. Expression of Mig and Crg-2 was detected in multiple organs and at relatively high levels in C57BL/6 mice after VV infection. Chemokine expression was detected by day three and generally peaked at around day six, which coincided with IFN-γ expression. Expression of IFN-γ, Mig and Crg-2 all decreased by day ten post-infection (Mahalingam *et al.*, 2000; Mahalingam and Karupiah, 2000a). Given the similarities between I-TAC, Mig and Crg-2, it would not be surprising if I-TAC is expressed and functions in a similar manner to these other chemokines during such infections.

4.1.2 Autoimmunity - EAE

Chemokine expression is not limited to viral or pathogen infections, indeed, expression of many chemokines has been detected during autoimmune diseases. During the CNS demyelinating disease, EAE, the infiltration of activated Th1 autoreactive T cells into the CNS occurs. Numerous chemokines are expressed during various stages of EAE and include; RANTES, MIP-1α, MIP-1β, MCP-1 and IP-10 (Hulkower *et al.*, 1993; Ransohoff *et al.*, 1993; Glabinski *et al.*, 1995; Godiska *et al.*, 1995; Glabinski *et al.*, 1997; Youssef *et al.*, 1998; Glabinski and Ransohoff, 1999). The exact role of these chemokines however, is still unclear.

Arimilli *et al* has previously used a monoclonal anti-CXCR3 Ab during a murine proteolipid protein peptide ($PLP_{139-151}$) induced EAE model. Injection of this Ab on days –1, +3, and +7 post disease induction demonstrated that mice were significantly protected from development of EAE (Arimilli *et al.*, 2000). This suggests a plausible use for therapeutic agents targeting the CXCR3 receptor for patients suffering from multiple sclerosis, and other similar autoimmune diseases (Arimilli *et al.*, 2000). This study indicates that Mig, IP-10 and I-TAC possibly enhance the severity of disease via CXCR3.

In addition to this, the high level of I-TAC expression in human astrocytes and its ability to attract activated T cells has led some to propose that I-TAC may have a very important role during the induction and pathophysiology of neuroinflammatory disorders including multiple sclerosis (MS) (Cole *et al.*, 1998; Luo *et al.*, 1998). Confirmation of a role in animal MS models, such as EAE, has been slow because a rodent I-TAC homologue had previously not been identified. Determining how a potent chemokine such as I-TAC might fit into the processes of autoimmunity is essential to thoroughly understand MS, EAE and perhaps-other inflammatory autoimmune diseases.

4.1.3 Transplantation

The migration of leukocytes into a transplanted graft is an essential requirement for graft rejection. Surgical trauma and ischemic damage during the early stages of transplantation and leukocyte infiltration can lead to further recruitment of excess effector leukocytes and hence the enhanced damage to the graft (Nelson and Krensky, 2001). The graft rejection process also relies heavily on the migration of DCs from the graft into secondary lymphoid tissue (Lakkis *et al.*, 2000). The migration of leukocytes into the transplanted region as well as the recruitment of DCs to lymphoid tissue therefore requires the activity of a multitude of chemokines being released throughout the transplantation process.

The chemokines Mig and IP-10 are expressed at high levels during acute cardiac allograft rejection, and CXCR3 deficient mice show profound resistance in the development of transplant rejection. One group of CXCR3 deficient mice were given a subtherapeutic course of cyclosporin A, an immunosuppressive agent, and subsequently showed permanent engraftment (Hancock *et al.*, 2000). In addition to this, mice with

normal CXCR3 function that were treated with anti-CXCR3 monoclonal antibody have shown delayed onset of allograft rejection (Hancock *et al.*, 2000). Since I-TAC also binds to the CXCR3 receptor, it is possible that I-TAC may also have an important function in transplant rejection.

Characterisation of I-TAC expression during different murine disease models may not only extend the understanding of the role of I-TAC during an immune response but could also help understand the immune processes during such diseases.

4.2 Materials and Methods

4.2.1 Mice

Specific pathogen-free 6-8 week old female C57BL/6, male BALB/c, and CBA/H mice were obtained from the Animal Breeding Facility, John Curtin School of Medical Research, Canberra, Australia. The origin of the IFN-$\gamma R^{-/-}$ and the age-matched null homozygote (C57/BL6 x 129/Sv) mice used for the EAE studies are described in section 3.2.1.

4.2.2 Virus disease models

4.2.2.1 RSV infection of mice

RSV strain A2 (ATCC number CCL-23) was maintained as described previously (Ghildyal *et al.*, 1999). BALB/c mice were infected intranasally with 1 x 10^5 PFU of RSV. Lungs were collected on days 0, 3 and 6, RNA isolated and cDNA generated before PCR analysis (section 2.2.5, 2.2.12 and 2.2.14).

4.2.2.2 Influenza A virus infection of mice

For influenza A (strain A/PR/8/34), C57BL/6 mice were infected intranasally with 200 HAU of virus as described previously (Karupiah *et al.*, 1998). Lungs were collected on days 0, 5, and 10 post infection and RNA isolated and cDNA generated before PCR analysis (section 2.2.5, 2.2.12 and 2.2.14).

4.2.2.3 VV infection of mice

The tissue culture adapted VV, VV-WR-L929-TK, referred to from hereon as vaccinia virus western reserve (VV-WR), was obtained and maintained as described previously (Boyle *et al.*, 1985). C57BL/6 mice were infected intranasally with 1 x 10^4 PFU of VV-WR. Lungs were collected on days 0, 3, 6, and 9 and RNA isolated and cDNA generated before PCR analysis (section 2.2.5, 2.2.12 and 2.2.14).

4.2.3 EAE model

The peptide corresponding to amino acids 35-55 of rat myelin oligodendrocyte glycoprotein (MOG - ^{35}Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val-

His-Leu-Tyr-Arg-Asn-Gly-Lys[55]) was synthesized by standard f-moc chemistry and the purity determined by reverse phase HPLC (Biomolecular Resource Facility, JCSMR, ANU). Rat MOG_{35-55} (2 mg/ml in saline) was emulsified in an equal volume of CFA containing 0.5 mg/ml *Mycobacterium butyricum* and 4 mg/ml *Mycobacterium tuberculosis* (H37Ra). Each mouse received 100 µl emulsion distributed in the foot pads of both hind feet and in the nape of the neck. Total dose of MOG_{35-55} was 100 µg/mouse. Immediately before, and 2 days after injection of the emulsion, the mice received an i.v. injection of 300 ng of pertussis toxin (List Biological Laboratory, Campbell, CA) in 250 µl of PBS. The mice were monitored daily and assigned EAE severity scores as described previously (Willenborg *et al.*, 1996). Briefly, phenotypic scores were given a rating from zero to five. Zero indicates no visible symptoms; 1, partial tail paralysis is observed; 1.5, moderate to full tail paralysis and partial hind limb paralysis; 2, tail paralysis and moderate hind limb paralysis; 3, complete paralysis of the hind limbs and tail and often associated with incontinence; 3.5, paralysis of the tail and hind limbs with partial fore limb paralysis; 4, total paralysis of the tail, hind and fore limbs; 5 indicates death. First strand cDNA was also produced from mRNA isolated from CNS tissue excised using standard techniques from mice with EAE on days 8, 14, and 20 post challenge.

4.2.4 Transplantation model

Transplantation models were set up as described in Simeonovic *et al.*, (Simeonovic *et al.*, 1999). Briefly fetal BALB/c mouse and fetal pig proislets (islet precursors) were prepared from BALB/c fetal pancreas (17 days gestation) and SLA^{dd} inbred fetal pig pancreas (68-76 days of gestation) by collagenase digestion (6mg/mL, Boehringer Mannheim, Germany), followed by culture in 10% CO_2 in air for 4-7 days. The BALB/c mouse proislets were used for the allograft transplantation model where as the pig proislets used for the xenograft transplantation model. Mouse and pig proislets were embedded in plasma clots prepared from recipient mice strain plasma ($1/20^{th}$ fetal pig pancreas equivalent of proislets/clot or 2 fetal mouse pancreas equivalents/clot). Six plasma clots carrying fetal mouse proislets or two plasma clots loaded with pig proislets were transplanted beneath the kidney capsule of recipient CBA/H mice. Mice were treated i.p. on days –1, 1, 3, 7 and 10 post transplant with either GK1.5 (anti-CD4) mAb (0.48mg Ig/150µL/dose) to generate a non-rejected graft or PBS (150µL/dose) to

generate a rejected graft. On days 4, 8 and 14, mice were killed and grafts collected for RNA extraction and first strand cDNA synthesis.

4.3 Results

4.3.1 Viral models

The interferon inducible chemokines IP-10/Crg-2, Mig and human I-TAC have previously been shown to be expressed in a number of different viral infections including RSV, influenza A and VV (Bussfeld *et al.*, 1998; Mahalingam *et al.*, 2000; Mahalingam and Karupiah, 2000a; Matikainen *et al.*, 2000; Zhang *et al.*, 2001). Given the potency of I-TAC and the ability for it to recruit cells during a CMI response, murine I-TAC expression was investigated in these three viral infections.

Peak RSV viremia occurs on days 5-6 and virus is cleared by day 10 (S. Mahalingam, personal communication.). The study carried out here showed that I-TAC expression corresponds to peak viremia with expression detected by RT-PCR on day 3 and increased levels on day 6 post infection compared to basal levels on day 0 (Fig 4.1A).

A high level of I-TAC expression was detected by RT-PCR on day 5 post infection with influenza A whereas only basal levels were detected on days 0 and 10 (Fig 4.1B). This coincides with most severe disease symptoms as observed by the mice being hunched up as well as slight shivering, as well as peak viremia as shown previously (Karupiah *et al.*, 1998).

Expression of I-TAC during a VV-WR infection was not detected by RT-PCR at any time point during disease (Fig 4.1C). Similar results were obtained after mice were injected i.p with higher lethal doses (1×10^6 PFU) of VV-WR (data not shown). This was surprising considering other published findings with other chemokines such as Mig and Crg-2 (Mahalingam *et al.*, 2000; Mahalingam and Karupiah, 2000a).

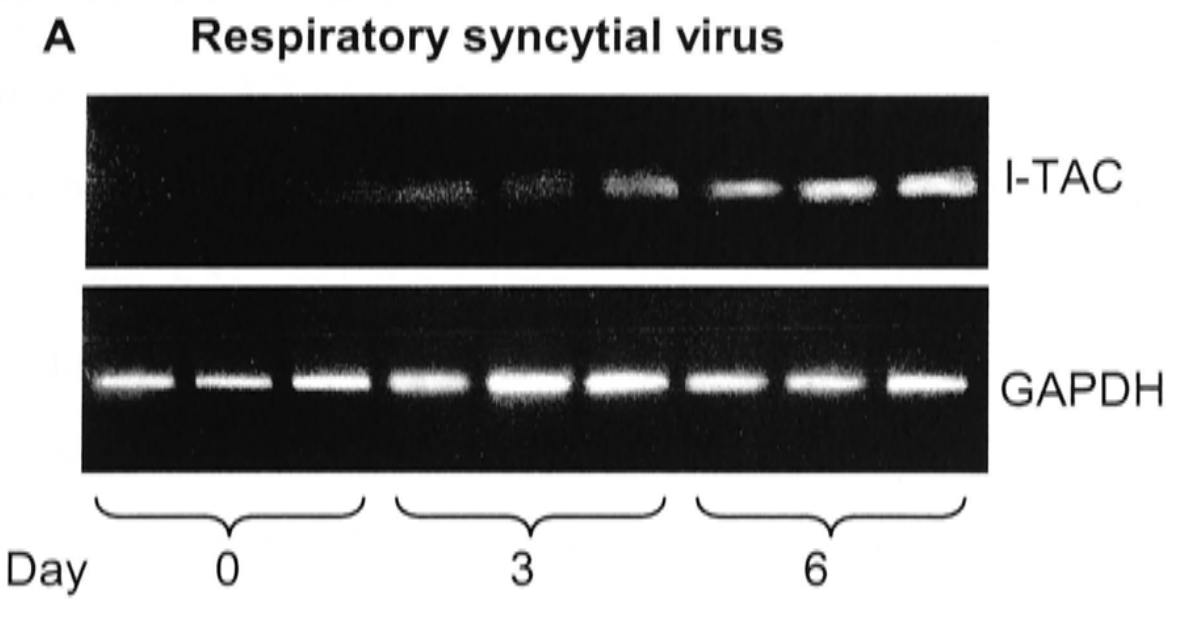


Figure 4.1: Analysis of murine I-TAC expression from mouse lungs during three different viral infections. BALB/c mice were injected intranasally with 1 x 10^5 PFU of respiratory syncytial virus (A) and samples analyzed on days 0, 3, and 6 post infection. C57BL/6 mice were injected intranasally with 200 HAU of influenza A virus (B) and samples analyzed on days 0, 5 and 10 post infection. C57BL/6 mice were injected intranasally with 1 x 10^4 PFU of VV-WR (C) and samples analyzed on days 0, 3, 6 and 9 post infection with a positive control.

4.3.2 Expression of murine I-TAC in the CNS of mice with EAE

Previously, it has been reported that IFN-$\gamma R^{-/-}$ mice challenged with rat MOG_{35-55} peptide develop severe EAE and die whereas wild type control mice (IFN-$\gamma R^{+/+}$) develop a milder disease and recover (Willenborg *et al.*, 1996). Given that HuI-TAC was isolated from astrocytes in the CNS, attracts activated T cells and is regulated by IFN-γ, murine I-TAC expression in the CNS of wild type and IFN-$\gamma R^{-/-}$ mice with MOG_{35-55}-induced EAE was investigated. Figure 4.2A shows the disease scores of the wild type and IFN-$\gamma R^{-/-}$ mice sacrificed on day 8 (before clinical disease), day 14 (peak disease in wild type mice) and day 20 (recovery in wild type mice). Semi-quantitative RT-PCR analysis detected very low levels of murine I-TAC in the CNS of IFN-$\gamma R^{-/-}$ mice throughout the time course (lanes 1-9) while these mice developed increasingly severe disease (Fig 2B). Analysis of wild type control mice however, showed that I-TAC levels in the CNS were initially low on day 8 (lanes 1-3), were very high at the peak of disease when the mice begin to recover on day 14 (lanes 4-6), but then returned to low levels by day 20 (lanes 7-9) (Fig 2C). These results do not provide a direct role for murine I-TAC during disease, however they measure I-TAC expression during EAE for the first time.

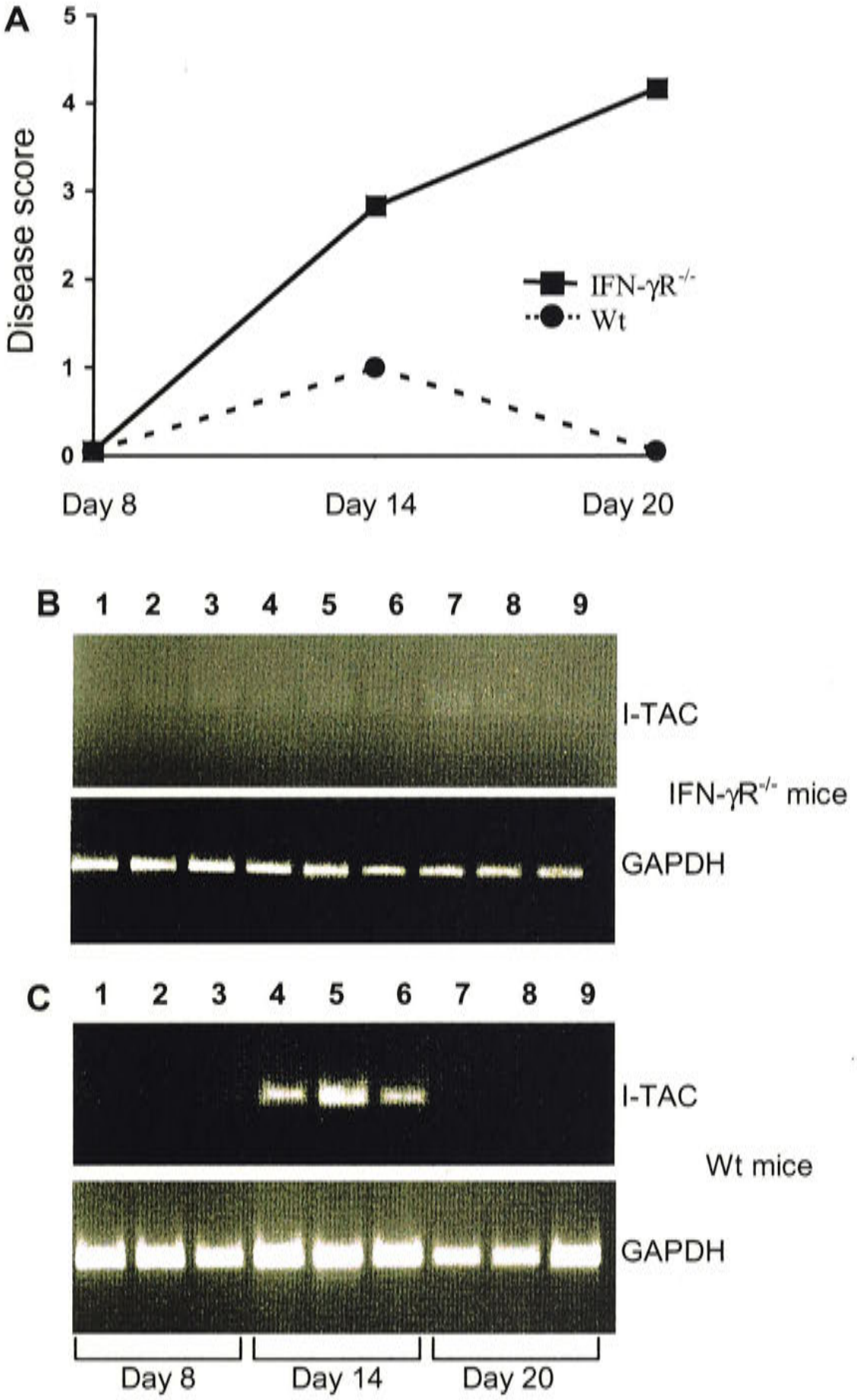


Figure 4.2: Analysis of murine I-TAC expression in the CNS of mice with EAE. A) Disease scores of wild type (circles) and IFN-$\gamma R^{-/-}$ (squares) mice with MOG_{35-55} peptide induced EAE. B) Semi-quantitative RT-PCR analysis of murine I-TAC and GAPDH mRNA CNS expression in IFN-$\gamma R^{-/-}$ mice with EAE at day 8 (lanes 1 to 3), day 14 (lanes 4 to 6) and day 20 (lanes 7 to 9). C) Semi-quantitative RT-PCR analysis of murine I-TAC and GAPDH mRNA CNS expression in wild type mice with EAE at day 8 (lanes 1 to 3), day 14 (lanes 4 to 6) and day 20 (lanes 7 to 9).

4.3.3 Transplantation model

Two different transplantation models were examined for I-TAC expression and therefore whether or not I-TAC has a possible role during the process of transplantation rejection. A murine allograft transplantation model was assessed, which uses a transplanted graft derived from a mouse of the same strain as the recipient. In addition to this, a xenograft model was also utilized where the transplant sample was derived from a fetal pig pancreas and grafted into a mouse recipient. In both cases non-rejected grafts were established by injecting anti-CD4 mAbs which inhibits CD4 T cell activity within the transplantation site.

Low levels of murine I-TAC mRNA expression are observed on days 4 and 8 in both rejected and non-rejected allograft samples but are absent on day 14 (Fig 4.3A). This shows that I-TAC is expressed from within the grafts most likely leading to the recruitment of T cells to the site of transplantation. As I-TAC is expressed in both rejected and non-rejected samples, expression is most likely a result of the transplantation process itself, causing general damage, inflammation and DC activation in the graft rather than as a result of allograft rejection or autoimmunity caused by T cells.

The results observed with the xenograft samples are less clear, with relatively high levels of I-TAC mRNA expression being observed in the rejected PBS treated mice, on day 4, none on day 8 and a moderate level on day 14. These changes in I-TAC expression may indicate a relapsing-remitting rejection process however the sample numbers are too low to be certain. A greater pool of sample numbers may establish a clearer trend. I-TAC expression was observed at high levels on days 8 and 14 from the non-rejected xenograft (anti-CD4 mAb treated) mice (Fig 4.3B). Recruitment of immune cells into the graft site is therefore likely to occur a week or so post operation, however the sample numbers are again to low to be confident of a significant trend. The results however, suggest that there must be cells, including DCs infiltrating the graft which subsequently express murine I-TAC, presuming that the RT-PCR is unable to amplify pig I-TAC, the sequence of which is unknown to date.

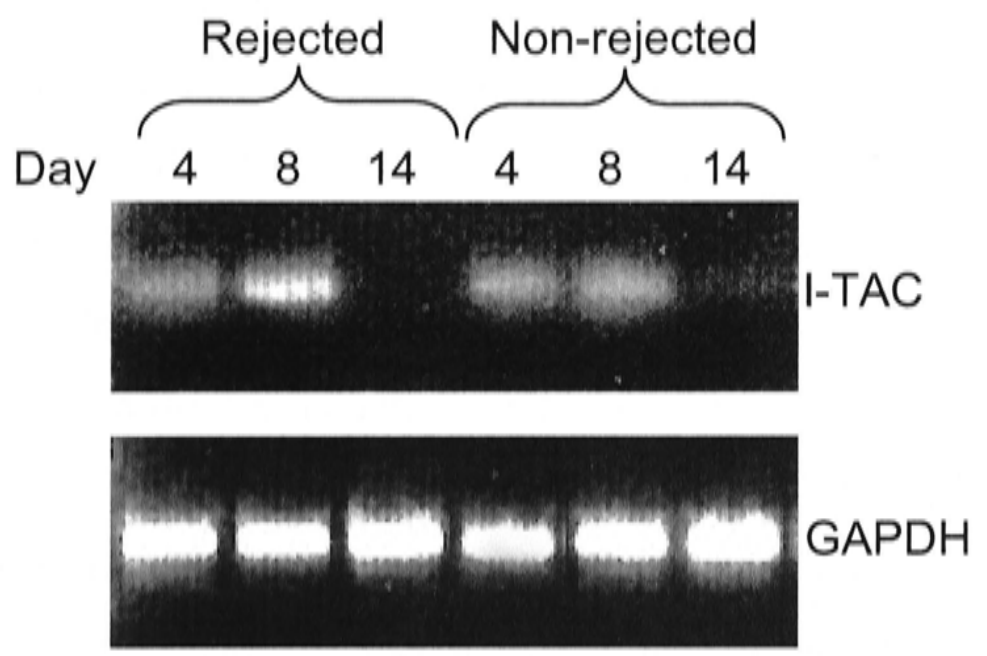


Figure 4.3A: Semi-quantitative RT-PCR of rejected and non-rejected allograft samples collected on days 4, 8 and 14.

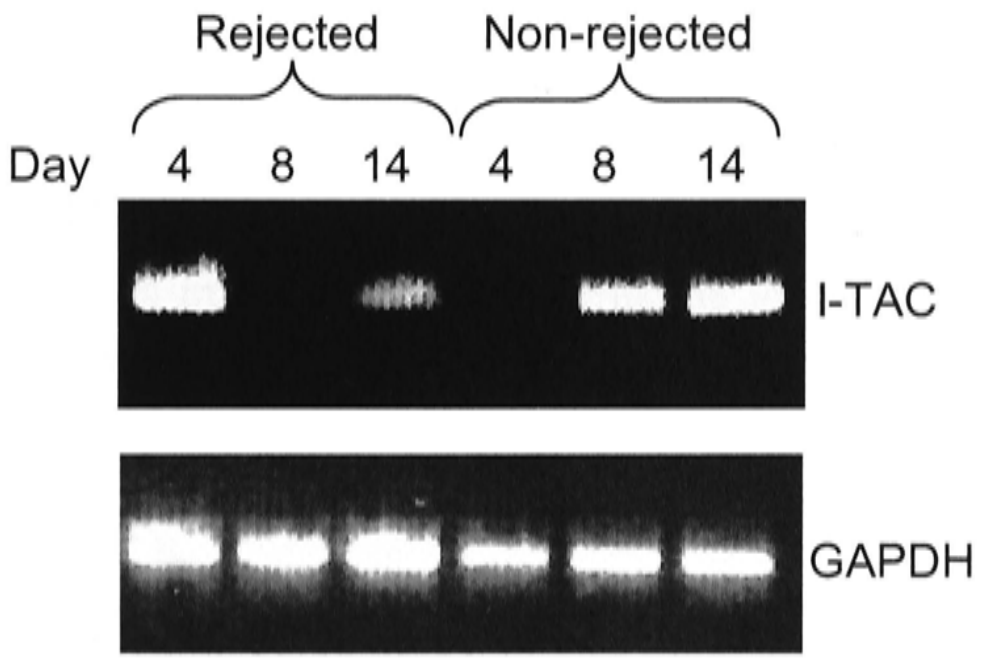


Figure 4.3B: Semi-quantitative RT-PCR of rejected and non-rejected xenograft samples collected on days 4, 8 and 14.

4.4 Discussion

4.4.1 I-TAC expression during viral infections

The induction of murine I-TAC from DCs, after stimulation with IFN-γ, suggests that I-TAC may have a role in CMI responses. Expression during different disease models was examined to observe if I-TAC expression was consistent with the known immune mechanisms functioning in each model. Many different viral infections including RSV, influenza A and VV can cause the induction of IP-10/Crg-2 and Mig (Bussfeld *et al.*, 1998; Mahalingam *et al.*, 2000; Mahalingam and Karupiah, 2000a; Matikainen *et al.*, 2000). Not surprisingly, human I-TAC expression has been detected during an RSV infection (Zhang *et al.*, 2001). The studies carried out here demonstrated that murine I-TAC was expressed during both RSV and influenza A viral disease models. Expression coincided around peak infection suggesting that murine I-TAC has a role during viral infection. I-TAC most probably functions by recruiting NK and Th1 cells to the site of infection and enhancing viral clearance and as a result may have an important role during CMI responses. The primary control of an influenza infection does not appear to require IFN-γ, although a lack of IFN-γ results in a significant reduction in cellular recruitment to the site of infection (Graham *et al.*, 1993; Baumgarth and Kelso, 1996; Karupiah *et al.*, 1998). This suggests that the main function for IFN-γ during an influenza infection is to regulate leukocyte trafficking, probably via I-TAC and the other IFN-inducible chemokines (Baumgarth and Kelso, 1996). Given the relative potency of I-TAC compared with the other IFN-inducible chemokines, artificially enhancing I-TAC expression in the lungs during either an influenza or RSV infection may reduce disease and be beneficial as part of a therapeutic regime. Inhibition of natural I-TAC expression by whatever reason, e.g. a genetic disorder, may make the disease worse, although there would presumably be some compensatory mechanism initiated by other chemokines.

I-TAC expression however, was not detected by RT-PCR in the VV-WR infection. This was somewhat surprising considering previous findings, which detected both Mig and Crg-2 in the liver, spleen, uterus and ovaries during similar experiments (Mahalingam *et al.*, 2000; Mahalingam and Karupiah, 2000a). In these previous studies expression levels of Mig, Crg-2 and IFN-γ however, were quite variable between these tissues, moreover, expression in lungs was not assessed. It may be that during an intranasal

infection of VV, DCs in the lung do not express I-TAC but instead mature and express this chemokine in the draining lymph nodes, which were not assessed. Also, recent findings in the vaccine immunology group, at the John Curtin School of Medical Research, have demonstrated that VV can kill immature DCs (from a DC line) very early during an *in vitro* infection (unpublished data). Hence if both of these effects operate *in vivo*, then there would be a reduced number of these cells capable of expressing I-TAC.

It is possible therefore, that the lack of I-TAC expression may be due to VV having a mechanism that inhibits I-TAC expression. Poxviruses including VV have developed several mechanisms to help evade the immune system (Lalani *et al.*, 2000). In fact, the genomes of most poxviruses appear to encode soluble cytokine and chemokine binding proteins (Puehler *et al.*, 1998). For example, the Lister strain of VV secretes a 35 kDa protein that inhibits eotaxin function. This chemokine binding protein also binds and inhibits activity of other CC chemokines including RANTES, MIP-1α, MCP-1, and I-309 (Alcami *et al.*, 1998). Poxviruses can also encode chemokine receptor homologues that also inhibit chemokine activity, for example, swinepox virus encodes a CXCR1 homologue, K2R reducing the effects of IL-8 (Cao *et al.*, 1995). Binding proteins or their receptors however, would probably not inhibit mRNA expression suggesting that inhibition of I-TAC in the assays here occurred upstream of initial expression.

VV encodes a soluble IFN-γR homologue, the B8R gene, which neutralizes the biological activity of IFN-γ in a number of species including man, cow, rabbit, rat and chicken, however it does not inhibit IFN-γ in the mouse (Goebel *et al.*, 1990; Alcami and Smith, 1995; Mossman *et al.*, 1995; Puehler *et al.*, 1998). Therefore, expression of I-TAC and the other IFN-inducible chemokines, Mig and Crg-2, may be inhibited directly by the B8R protein in these other animals but this could not be the case in mice. However, it has recently been demonstrated that VV-WR can inhibit IFN-γ signal transduction (Najarro *et al.*, 2001). The mechanism utilized by VV-WR is independent of the B8R gene blocking IFN-γ activity and instead uses a viral phosphatase, VH1, encoded by VV-WR. The activity of VH1 inhibits phosphorylation and subsequent nuclear translocation of the STAT1 transcription factor. Consequently, this blocks expression of mRNAs normally induced by IFN-γ (Najarro *et al.*, 2001). This study also

demonstrated that the inhibition of STAT1 by VH1 does not alter the activity of upstream signalling molecules such as JAK1 and JAK2. As STAT1 is a crucial component of the major IFN-γ pathway, it is likely that inhibition of STAT1 by VH1 inhibits I-TAC expression during VV-WR infection in mice. The expression of Mig and Crg-2 during a VV infection may be due to an alternative signalling pathway, not requiring STAT1.

Inhibition of NF-κB, a transcription factor involved in the IFN-γ, CD40 and TNF-α signalling pathways, by poxviruses has also been demonstrated (Bowie *et al.*, 2000; Gil *et al.*, 2001; Oie and Pickup, 2001). The strain of virus appears to be important for NF-κB inhibition, for example Cowpox, molluscum contagiosum, racoonpox, VV Copenhagen strain and VV-WR can inhibit NF-κB activity. In contrast the modified VV Ankara strain, an attenuated virus, lacks the ability to inhibit NF-κB (Bowie *et al.*, 2000; Gil *et al.*, 2001; Oie and Pickup, 2001). The lack of NF-κB activity induced by poxviruses occurs by components expressed by the viruses (i.e. SalF9R and SalF15R genes expressed by VV-WR), which interfere with the degradation process of IκBα, a negative regulator of NF-κB (Oie and Pickup, 2001). As a result of VV-WR inhibiting NF-κB it is possible that murine I-TAC may not subsequently be expressed during infection. NF-κB activation can also induce Mig and IP-10 expression (Ohmori and Hamilton, 1995; Ohmori *et al.*, 1997). It would be expected then, that NF-κB inhibition by VV-WR may reduce or inhibit expression of these two chemokines compared to other infections such as RSV or influenza A.

Inhibition of I-TAC and other chemokine expression by poxviruses such as VV and variola (smallpox) may explain why these viruses are such successful pathogens. Artificially expressing I-TAC at the site of a VV infection, and possibly other poxvirus infections, may inhibit this immune evasion mechanism and aid viral clearance and recovery.

4.4.2 I-TAC expression during EAE

It has been speculated that human I-TAC, expressed from astrocytes and other APCs in the CNS, can exacerbate neuroinflammation and autoimmunity because it can recruit activated T cells and appears to be an early mediator of immune responses (Cole *et al.*,

1998; Luo *et al.*, 1998). The expression of murine I-TAC during EAE was examined to see if it had a role in this disease model. Analysis of wild type mice with MOG peptide-induced EAE demonstrated that murine I-TAC expression was highest at the peak of disease severity when the animals first begin to recover. This was somewhat later than expected and suggests that I-TAC does not have a role in the early disease processes. This is consistent with a previous study that showed that the main function of the currently known EAE-associated chemokines is to regulate later CNS inflammatory reactions as opposed to aiding the initial entry of autoreactive T cells into the CNS (Glabinski *et al.*, 1999).

Recently, Willenborg *et al*, reported that IFN-γ $R^{-/-}$ mice with MOG peptide-induced EAE develop severe disease and die whereas wild type mice develop mild disease and recover (Willenborg *et al.*, 1996). The report by Willenborg *et al*, demonstrated that IFN-γ, while primarily a proinflammatory cytokine, is also important for disease recovery. This study found that I-TAC expression was very low at all stages of EAE disease when induced in IFN-γ$R^{-/-}$ mice. These results do not provide a direct link between the function of murine I-TAC and the EAE disease phenotype. It is however, interesting to speculate, given the lack of murine I-TAC in the IFN-γ$R^{-/-}$ mice that I-TAC might have a role during disease recovery in wild type mice by recruiting suppressor T cells. It is more likely however, given that antibodies to a functional domain of CXCR3 protect mice from EAE, that murine I-TAC acts to enhance neuroinflammatory processes (Arimilli *et al.*, 2000). As there are other chemokines that bind to CXCR3, a thorough assessment of the contribution of murine I-TAC in the progression of neuroinflammation however, remains to be investigated.

4.4.3 I-TAC expression during two graft transplant models

The migration of leukocytes into the regions of transplanted grafts as well as the recruitment of DCs to lymphoid tissue requires the activity of numerous chemokines. Characterising the chemokines involved could aid in the development of therapeutic agents to block leukocyte infiltration and ultimately inhibit the rejection process. I-TAC mRNA expression is observed throughout the early to mid stages of allograft transplantation (in both rejected and non-rejected samples). This expression pattern is unlike the viral models or the EAE transplantation model indicating that I-TAC may be

expressed as a result of general damage following transplantation surgery rather than as a direct result of specific allograft rejection or autoimmunity. Previous studies have examined the expression of IFN-γ during transplantation models (Morris *et al.*, 1995; Simeonovic *et al.*, 1999). Expression of IFN-γ appears around day five during rejected allografts although there is very little expression during non-rejected allografts (Simeonovic *et al.*, 1999).

The study analyzing tissues from rejected xenograft transplantation showed varying results throughout the time course, which may indicate differing levels of inflammation and cellular recruitment in different mice. The necessity of murine DCs to migrate into the graft before beginning to express chemokines may explain why murine I-TAC was not expressed until day 8 in the non-rejected samples. The nature of the transplantation models used here however, meant that only one mouse sample was taken at each time point. This means that variation between individual mice could not be addressed and determining whether I-TAC expression or indeed any other chemokine was important was difficult. Although these results do not appear to show a clear trend of I-TAC expression in the different transplantation models they perhaps show that cells expressing I-TAC migrate into the graft provided there is no RT-PCR cross-reactivity to pig I-TAC which is unlikely. These cells may be DCs or other APCs, which then express I-TAC in order to recruit T cells. Utilizing anti-CD4 mAbs to block rejection did not clarify the situation with respect to I-TAC expression. Anti-CD4 mAbs inhibit CD4 cell activity resulting in the inhibition of the rejection process however, this would not alter I-TAC expression from APCs from within the transplanted graft. Inhibiting early events such as host APCs from infiltrating transplantation grafts may help reduce the rejection process. Blocking chemokine receptors, including CXCR1, CCR1 and CCR2, which are expressed on APCs during early migration events, may be one way of achieving this. Recently it has been demonstrated that the administration of anti-CD40L Ab to non-human primates can prevent rejection of renal allografts (Kirk *et al.*, 1999). The mechanism for rejection inhibition is still unclear, however T cell infiltration still occurs (Kirk *et al.*, 1999). The lack of CD40 activity may halt $CD4^+$ T cell activation required before rejection can be initiated.

Many studies have demonstrated that CXCR3 and $CD4^+$ T cell activity are important during the rejection process and indeed, inhibition of T cell activation or mononuclear migration has been shown to inhibit or at least delay the onset of the rejection process (Russell *et al.*, 1996; Chandraker *et al.*, 1998; Melter *et al.*, 1999; Hancock *et al.*, 2000). As such, blocking the activity of individual chemokines like I-TAC, as well as Mig and IP-10 may be a useful therapeutic tool to inhibit or delay the rejection process. It is probably more practical however, to block the CXCR3 receptor which binds all three chemokines as has previously been demonstrated (Hancock *et al.*, 2000).

These studies have confirmed that I-TAC is expressed during immune responses towards disease situations. The outcome of I-TAC activity most likely depends on the disease in question. I-TAC expression during viral infections probably supports viral clearance by attracting NK and Th1 cells to the site of infection, whereas during EAE, I-TAC may exacerbate disease by recruiting autoreactive T cells into the CNS. To further analyze the *in vivo* function of I-TAC during disease challenges several methods could be applied. Two commonly approved methods to deregulate and/or overexpress a gene of interest are by utilizing either DNA plasmid vaccines or recombinant viral vectors.

CHAPTER 5.

MODULATION OF IMMUNE RESPONSES USING I-TAC

CHAPTER 5. MODULATION OF IMMUNE RESPONSES USING I-TAC

5.1 Introduction

Expression of I-TAC from DCs in response to IFN-γ (chapters 2 and 3), and during different Th1 biased disease models (chapter 4) suggested that I-TAC may have an important role during induction and/or recall of cell-mediated immune responses. Exploiting chemokines for therapeutic agents or using them to augment vaccines has been postulated and tried in several different model systems (Youssef *et al.*, 1998; Biragyn *et al.*, 1999; Narvaiza *et al.*, 2000; Stoof *et al.*, 2001). Utilizing I-TAC to attempt to modulate the immune response during disease not only assesses its potential for therapeutic and/or vaccine agents but also aids the further understanding of its functions *in vivo*.

5.1.1 Recombinant VV encoding I-TAC

An approach commonly used in conjunction with transgenic and gene knock out mice to study the *in vivo* effects of cytokines and chemokines is to engineer recombinant viruses that express the cytokines or chemokines of interest. These constructs are then utilized to study the effects of these factors on host immune responses during virus infection. During replication *in vivo*, the recombinant virus produces the encoded factor, which is then secreted from infected cells. The location and extent of virus replication determines the levels and sites of cytokine or chemokine production. This approach enables the effects of these molecules on the development of both the innate and adaptive immune responses mounted against the virus infection to be studied *in vivo*.

Many of the encoded factors, including the cytokines IL-2 and IL-12, and the chemokines Mig and Crg-2, have demonstrated potent antiviral activity *in vivo* (Flexner *et al.*, 1987; Ramshaw *et al.*, 1987; Yilma *et al.*, 1987; Ramshaw *et al.*, 1997; Hogan *et al.*, 1998; Mahalingam *et al.*, 1999). Each of these factors induced particular antiviral host defense mechanisms, whilst for other encoded factors such as, IFN-γ, TNF-α and CD40 ligand, a direct antiviral effect was demonstrated (Kohonen-Corish *et al.*, 1990; Sambhi *et al.*, 1991; Ruby *et al.*, 1995). IL-12, which also showed potent antiviral activity when expressed by rVV, is a strong stimulator of IFN-γ production. As early as

one day after infection, VV encoding IL-12 was recovered at titres approximately 100-fold lower than a control rVV in normal mice. However, IL-12 itself may also exert antiviral activity, as demonstrated by the observation that mice deficient in this factor failed to mount significant antiviral CTL responses or to clear the virus efficiently, despite unimpaired IFN-γ expression (van Den Broek *et al.*, 2000). Thus, in addition to its regulatory activity in Th1 cell development, IL-12 may also play an important role as an antiviral mediator, both through the activity of IFN-γ and independently of this factor (Banyer *et al.*, 2000; Banyer *et al.*, 2002 submitted, see appendix 1c).

Expression of the T cell surface molecule CD40 ligand in rVV also produced, somewhat unexpectedly, a highly attenuated virus phenotype *in vivo* (Ruby *et al.*, 1995). The attenuation of rVV-CD40 ligand in wild type mice was, however, completely reversed in CD40-deficient mice. Interestingly, CD40 expression is not restricted to B cells, macrophages and DC, but is also found on tumour cells and may be induced on fibroblasts incubated with IFN-γ and TNF-α. Thus, it has been speculated that CD40 ligand/T cell-mediated activation of CD40 on virus-infected cells may act to limit the production of progeny virus, possibly through apoptosis of infected cells (Ruby *et al.*, 1995).

Using this recombinant VV expression system, Mig and Crg-2 have been shown to exhibit antiviral activity *in vivo* (Mahalingam *et al.*, 1999). In infected mice, rVV-encoded Mig or Crg-2 (rVV-Mig or rVV-Crg-2) enhanced the cytolytic activity of natural killer (NK) cells, mononuclear cell infiltration in livers and IFN secretion by two- to threefold and resulted in significant viral clearance and survival in nude mice. In contrast, mice infected with a control virus (rVV-X) that did not express a chemokine succumbed to generalized infection (Mahalingam *et al.*, 1999).

Construction of a recombinant VV encoding I-TAC would enable studies similar to the Mig and Crg-2 experiments to be carried out. The results obtained would enable further understanding of the function of murine I-TAC *in vivo*.

5.2 Materials and Methods

5.2.1 Mice

Five to eight week old female C57BL/6 (wild-type) mice, and athymic, Swiss nude outbred mice were bred under specific pathogen free (SPF) conditions and obtained from the ABE, JCSMR.

5.2.2 Cell lines

A human osteosarcoma cell line, 143B, (Rhim *et al.*, 1975) were maintained in F15 minimum essential medium (MEM) supplemented with 5% heat inactivated foetal calf serum (FCS; Flow laboratories, North Ride, Australia), 1 mM L-glutamine (Gibco), 10 mM HEPES (Sigma), and antibiotics (PSN;): 30 µg/mL penicillin G, 50 µg/mL streptomycin sulphate and 50 µg/mL neomycin sulphate (all from Sigma). 143B cells were incubated at 37°C, in 5% CO_2 in air.

5.2.3 Histology

Organs were removed and immediately fixed in 10% neutral buffered formalin solution, processed and embedded in paraffin wax. Tissue sections (5µM thick) were mounted on glass slides and stained with hematoxylin and eosin (H and E) in the Histology facility, JCSMR.

5.2.4 Plasmid cloning

Cloning of target cDNA into the plasmid, pTK7.5A (Khanna *et al.*, 1991) was carried out using methods described in section 2.2.7 and using the BamH1/Sal1 restriction enzyme sites. Plasmid DNA was grown and prepared as described in section 2.2.9.

5.2.5 Recombinant VV construction

A recombinant VV construct was made by inserting the target I-TAC cDNA into the multiple cloning site of the shuttle plasmid pTK7.5A (sections 2.2.7 and 5.2.4). This plasmid contains the thymidine kinase (tk) gene following the PF vaccinia promoter as well as the P7.5 early/late promoter upstream of the multiple cloning site. The cloned plasmid was used to generate a recombinant VV by marker rescue recombination with the tissue culture adapted VV, VV-WR-L929-TK with the thymidine kinase gene region

previously disrupted by recombination with another plasmid, pBCB07 (Boyle *et al.*, 1985; Coupar *et al.*, 1988).

The parent VV WR-L929 tk$^-$ virus was trypsin treated for 30 min at 37°C (100 µL virus to 100 µL 1 mg/mL trypsin) and the reaction stopped by adding 800 µL 5% FCS F15 MEM media. L929 cells were infected with 0.01-0.05 multiplicity of infection (MOI) of virus for 1 hr and covered with 5% FCS F15 MEM media. Plasmid DNA was precipitated by making up dilutions of 10, 20 and 40 µg DNA in 20 µL MQW, and 2 µL calf thymus DNA (10 µg/mL) and 1 mL Hepes buffered saline (pH 7.0). 65 µL of 2M $CaCl_2$ was added dropwise, then allowed to stand for 45 min. Media was removed from infected cells and plasmid DNA precipitate added evenly and left at room temp for 30 min. Fresh 5% FCS F15 MEM media was then added and left at 37°C, 5% CO_2 for 6 hrs. Media was then replaced with 1 x MTAGG (3 µM Methotrextate; 15 µM Thymidine; 50 µM Adenosine; 50 µM Guanosine; 100 µM Glycine, made up to 1 mL MQW) in 5% FCS F15 MEM media and incubated at 37°C 5% CO_2 for two days.

Amplification of virus stocks was then carried out. Cells were collected by scraping and centrifuged (5 min, 3000 rpm) and resuspended in PBS. 100 µL of the virus was trypsin (100 µL of 1 mg/mL) treated for 30 min at 37°C and the reaction stopped by adding 800 µL 5% FCS F15 MEM media, then added to a small flask of 50% confluent 143B cells. Media containing MTAGG selection was added 1 hr later and grown for two days.

5.2.6 Plaque purification

An aliquot (100 µl) of the amplified virus stock was trypsinized with 100µL trypsin (1 mg/mL) in PBS and was incubated at 37°C for 30 min. A range of dilutions of the virus stock were used to infect 143B cells grown in 6 well cluster plates for 1 hr before media containing FCS was added. The titration was carried out using 10^{-1} to 10^{-6} dilutions under selection conditions (2 mL selection media/well containing MTAGG). After two days of incubation at 37°C, 5% CO_2 in air, the medium was removed by aspiration, individual plaques picked in 2-3 µl PBS and used to infect fresh 143B cells grown in 24 well plates (Flow Laboratories). The virus was incubated for 1 hr at 37°C before 1 mL/well of selection medium was added and the plate incubated for a further 2 days at

37°C. The media was removed from each well of cells and 50 µL PBS added. Each plate was stored at –20°C until screened for the presence of the cloned I-TAC gene.

5.2.7 Detection of recombinant VV by dot blot hybridization

To screen for the presence of the inserted gene, 5µL of the virus samples were dotted onto Hybon-N^+ nylon membranes (Amersham) and allowed to dry. The membranes were placed once on 0.5M NaOH soaked 3 MM Whatman paper for 5 min, followed by three washes on 1 M Tris (pH 7.5) soaked 3 MM Whatman paper, each for 5 min, then twice on 0.5 M Tris (pH 7.5), 1.5 M NaCl soaked 3 MM Whatman paper for 5 min. The membranes were fixed by UV exposure then incubated at 68°C in prehybridization solution (5x SSPE, 0.2% SDS, 0.2% skim milk powder, 0.2mg/mL salmon sperm DNA) for 4 hrs. The membrane was then hybridized for 24hr at 68°C. The hybridization mix was similar to the prehybridization mix except it contained a radio-labelled cDNA probe. I-TAC cDNA was labelled for hybridization with 5µL α^{32}P-dCTP (Bresatec) using the Ready-To-Go labelling system (Pharmacia), according to the manufacturer's protocol. Labelled cDNA was purified by passage through a Nick Column (Pharmacia). Membranes were then washed twice in 2x SCC, 0.1% SDS for 15 min, one wash in 0.5x SCC, 0.1% SDS for 15 min and one in 0.1x SCC, 0.1% SDS for 15 min to eliminate non-specific hybridization. The membranes were then blotted dry, sealed in a plastic bag and autoradiography was carried out at –70°C using XAR5 X-ray film (Kodak).

5.2.8 Detection of rVV and absence of wild-type VV by PCR

The presence of the inserted gene was detected by dot blot hybridization and the absence of wild-type VV in the plaque purifications was confirmed by PCR. Viral DNA was extracted as previously described (Heine and Boyle, 1993). A small volume (~5-10 µl) of 143B cells from 24 well plates (described in section 5.2.2) was mixed with 0.5 mg/ml Proteinase K in 100 µl lysis buffer (10 mM Tris-HCL, 50 mM 2-mercaptoethanol, 100 mM NaCl, 10 mM EDTA, 1% N-Lauroylsarcosine, 26% sucrose, pH 7.6) and incubated for 1 hr at 56°C. DNA was then extracted and precipitated using the phenol/chloroform and ethanol precipitation (section 2.2.7). The DNA was resuspended in 20 µl H_2O and a PCR was carried out on 2 µL of the template DNA for both the I-TAC gene (described in section 2.2.5) and the wild-type VV. The primers used for the wild-type VV were; F region sense 5'- GGTTAATATGACGCTCG -3' and

antisense 5'- GCGTCACAGAATCTACC -3'. The PCR thermal cycle was as described in section 2.2.5 with an annealing temperature of 42°C for 30 cycles.

5.2.9 Amplification of virus stocks and storage

Once a suitable recombinant virus clone was identified, both the rVV I-TAC and VV-WR viruses were amplified by growing the viruses in 75 cm^2 tissue culture flasks on 143B cells. These cells were then used to seed 143B cells growing in 2L roller bottles (Corning Inc., USA) in media without selection. The infected cells were harvested 2 days later by scraping the cells into the media (with the use of a cell scraper), and the media centrifuged at 3,000 rpm for 5 min. The pellets were resuspended in 10mL gelatin saline with 2 mM HEPES and aliquoted into sterile 1.5mL ampoules and stored at 70°C.

5.2.10 Titration of virus

To titrate viral samples and stock solutions, 100µL of sample was trypsinized by incubating with 100µL trypsin (1mg/ml) at 37°C for 30 min. The trypsin was then neutralized by adding 800µl of F-15 MEM, 5% FCS. Serial 10-fold dilutions were then made in F-15 MEM (no FCS added) and used to infect 143B cells grown in 6-well tissue culture plates (described in section 5.2.2) for 1 hr, after which the cells were overlaid with media (F15 MEM, 5% FCS). Two days after incubation at 37°C the media was removed and the cells were fixed and stained with 2mL of 0.1% crystal violet and 20% ethanol in MQW for 5 min. The plaques were counted and the plaque forming units per mL (PFU/mL) of viral supernatant were calculated.

5.2.11 Chemotaxis detection of I-TAC expression by rVV

Functional chemokine protein expression by the recombinant VV was determined by chemotaxis assay. Supernatant from 143B cells infected with either VV-WR or rVV I-TAC was collected 24 hrs post infection and concentrated approximately 4 fold (described in section 2.2.11) and used in a chemotaxis assay using Con A activated splenocytes as described in sections 2.2.12 and 2.2.13. This assay was repeated multiple times to confirm the data.

5.2.12 Determination of virus titres in ovaries

Ovaries were removed aseptically from groups of 5 C57BL/6 mice infected with either VV-WR or rVV I-TAC (1×10^6 PFU i.p), on day 6 post-infection, and immediately frozen in dry ice and stored at –70°C. The ovaries were homogenized in 1mL of PBS. 100 µl of homogenate was incubated for 30 min with 100 µl trypsin (1 mg/ml) at 37°C. Ten-fold serial dilutions were made in F15 MEM media and 100 µl of each dilution was added to 143B cell monolayers grown in six-well cluster plates (Flow laboratories). After incubation for 48 hrs at 37°C, 5% CO_2, the monolayers were stained with 0.1% crystal violet in 20% ethanol for 5 min and the plaques enumerated.

5.2.13 Mortality studies

Groups of six C57BL/6 and athymic Swiss nude mice of the age of five weeks old were infected i.v with varying doses ($1 \times 10^4 - 1 \times 10^7$ PFU) of either VV-WR or rVV I-TAC and their progress monitored daily. Mice with severe symptoms including, shaking, severe weight loss, mangy fur, reduced activity and lack of responsiveness to external stimuli were euthenised and one day added to their mortality recording.

5.2.14 Foot pad analysis

Both hind footpads of naïve mice and preimmunized mice (1×10^5 PFU, 5 weeks before challenge) were injected with 1×10^7 PFU (in 20µL) of either VV-WR or rVV I-TAC. Footpad thickness was measured daily using calipers. Basal footpad thickness (measured prechallenge) is standardized as zero inflammation. Footpads were also removed from euthanised mice 24 hrs post infection and fixed in 10% neutral buffered formalin solution and embeded in paraffin wax (section 5.2.3). Footpad sections were cut perpendicular to the sole of the footpad and stained with H and E for histology analysis (section 5.2.3).

5.3 Results

5.3.1 Construction of a recombinant VV encoding I-TAC (rVV I-TAC)

Studies examining VV encoding the chemokines Crg-2 and Mig demonstrated an attenuation of this virus due to enhanced recruitment of NK cells and T lymphocytes (Mahalingam *et al.*, 1999; Mahalingam and Karupiah, 2000a). To assess if I-TAC has a similar effect, a shuttle vector plasmid, pTK7.5AI-TAC (Fig 5.1) was constructed and used to generate a TK^+ recombinant VV encoding murine I-TAC using marker rescue recombination. The VV-WR-L929-TK^+ virus (referred to from here on as VV-WR) was used as a control throughout all following experiments as rVV I-TAC is a TK^+ virus due to the construction and selection process. Functional protein expression of this chemokine by rVV I-TAC was demonstrated by carrying out a chemotaxis assay, using VV-WR as a control (Fig 5.2). Chemotactic activity by rVV I-TAC was similar to that as described in chapter two while VV-WR samples were consistent with the media only control. The similarity between this assay and the baculovirus based expression system (chapter 2) is not surprising given that both assays use the concentrated protein preparation in the same F15 media contained in the chemotaxis chambers.

5.3.2 Viral titres in C57BL/6 mice

To test the immune response against VV-WR and rVV I-TAC, a number of different analyses were carried out. Initially ovaries were isolated from mice infected with either virus on day 6 and viral titres enumerated. Ovaries were chosen as this organ is a sensitive indicator of viral growth and subsequent immune responses against the virus (Karupiah *et al.*, 1990). The average titre for mice infected with either VV-WR or rVV I-TAC was $(1.7 \pm 0.18) \times 10^6$ and $(1.5 \pm 0.25) \times 10^3$ respectively. These results were significantly different ($p \leq 0.05$, student's t-test). The lower viral titre indicated that expression of I-TAC significantly inhibited the growth of the virus *in vivo*.

5.3.3 Mortality studies in VV infected mice

To assess whether lower titres in ovaries influenced the overall immune response against VV infection, mortality studies were carried out. Mortality studies of VV-WR and rVV I-TAC of five week old C57BL/6 and athymic Swiss nude mice were assessed. Groups of six female mice were injected i.v. with varying doses of either VV-WR or rVV I-TAC and observed for 30 days. The doses ranged from 1×10^4 to 1×10^7 PFU. Figure 5.3 shows the mortality rate of C57BL/6 mice at different infection doses.

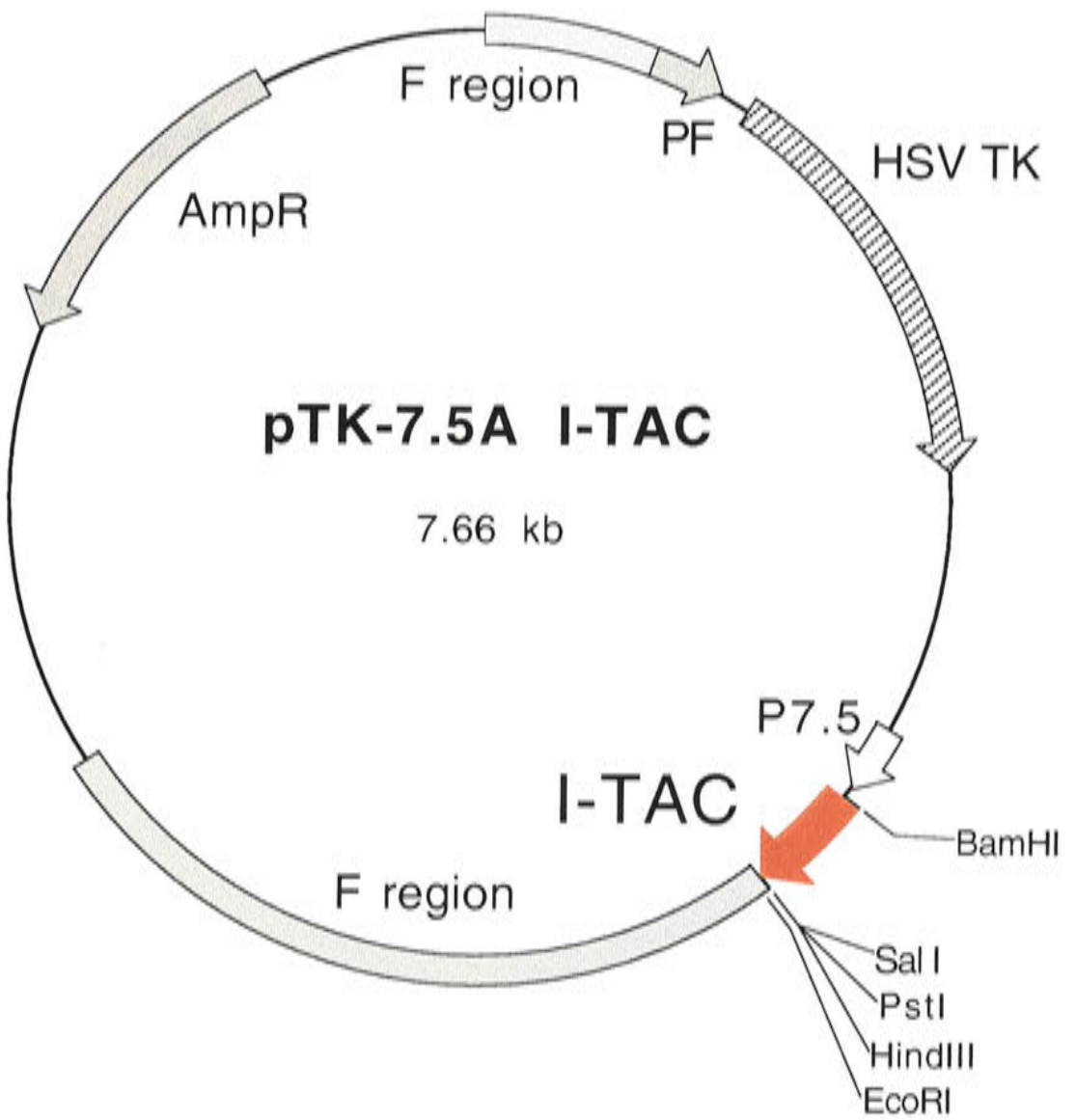


Figure 5.1: Construction of insertion plasmid pTK7.5-I-TAC. A BamHI and Sal I fragment containing I-TAC was excised from bluescript. The gene fragment was then subcloned into pTK7.5A at the BamHI and Sal I sites located behind a P7.5 promoter (Coupar *et al.,* 1998). AmpR, ampicilin resistance gene; PF, vaccinia promoter; HSV TK, thymidine kinase gene of herpes simplex virus; P7.5, vaccinia early/late promoter.

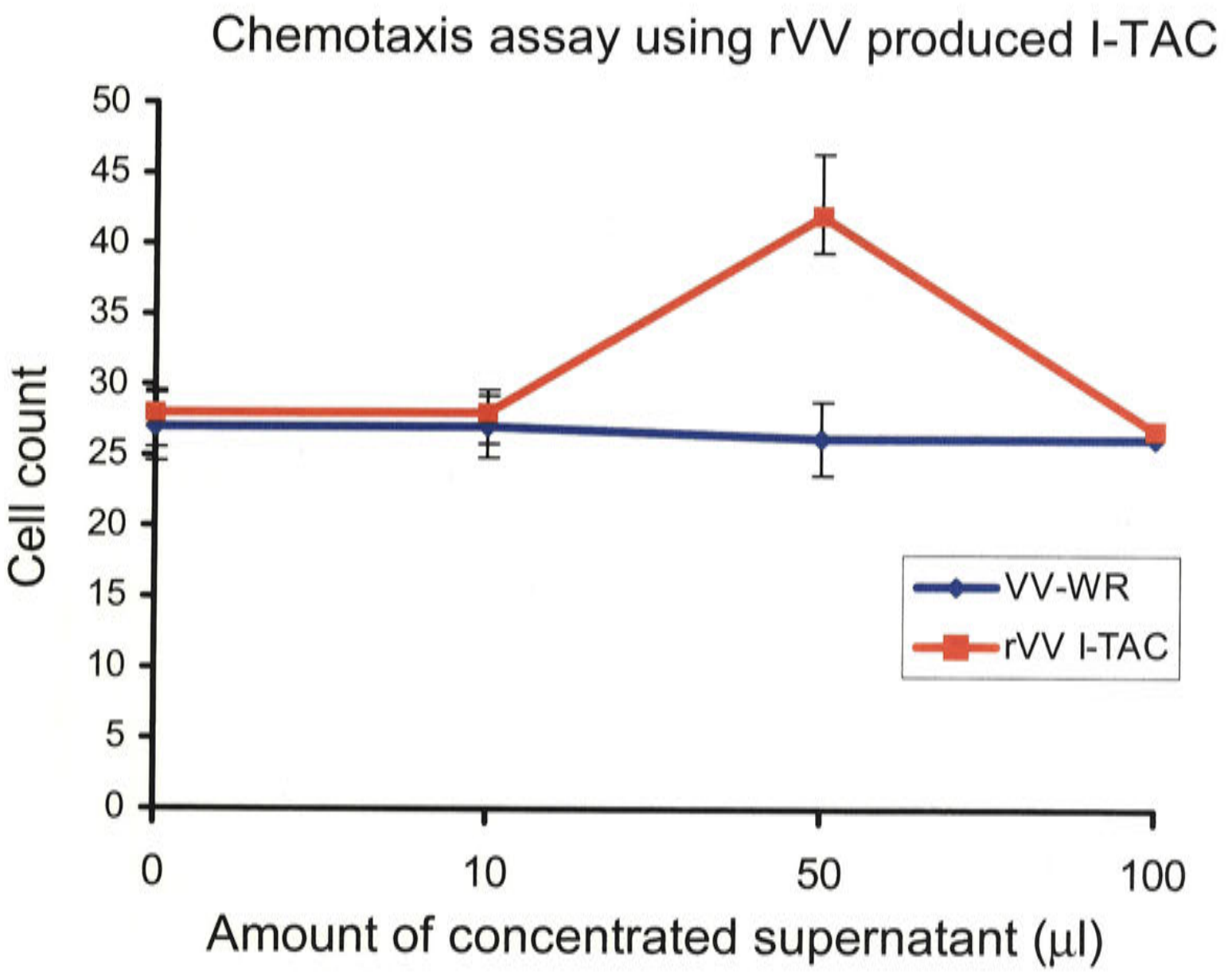


Figure 5.2: Chemotaxis assay using rVV produced I-TAC. Supernatant from VV-WR or rVV I-TAC infected 143B cells was collected and concentrated ~4 fold and used for a chemotaxis assay as described in chapter 2. Con A blasted splenocytes (24 hrs stimulation) from naïve C57BL/6 mice were used for the assay. Standard deviation is shown and there is a significant difference between rVV I-TAC and both VV-WR at the 50 µL supernatant amount and media only samples (student's t-test $p<0.0002$).

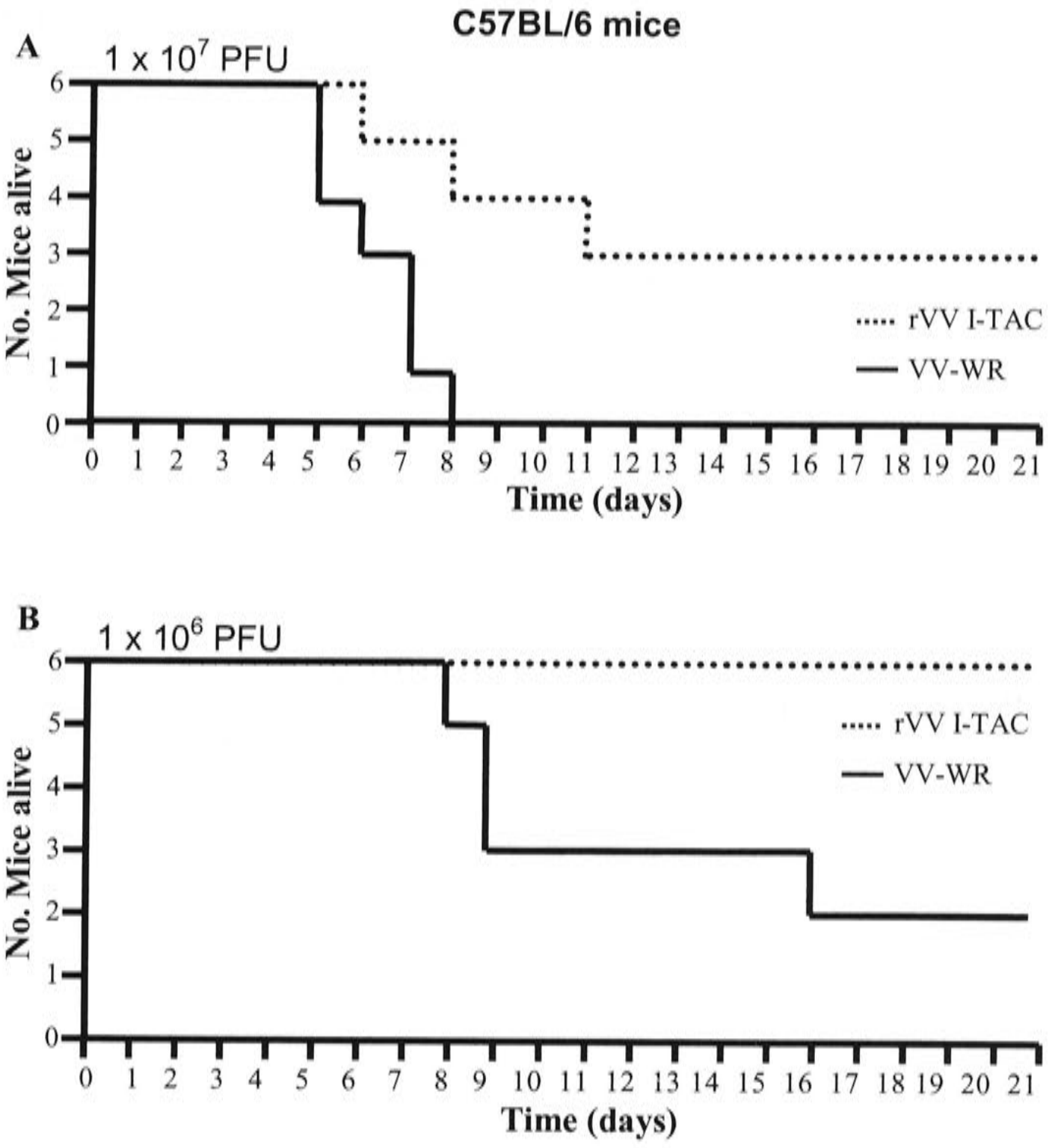


Figure 5.3: Mortality studies. Groups of 6 C57BL/6 mice were challenged i.v. with either VV-WR or rVV I-TAC at varying doses with their time of death being recorded. Doses used were 1 x 10^7 PFU (A), 1 x 10^6 PFU (B), 1 x 10^5 PFU (not shown) and 1 x 10^4 PFU (not shown). All mice in the last two challenge doses survived. Solid lines (——) represent mice infected with VV-WR, whereas dashed lines (·····) represent mice infected with rVV I-TAC.

Most C57BL/6 mice were able to resolve infection at doses of 10^7 PFU challenged with rVV I-TAC and all mice survived at lower doses whereas all mice succumbed to VV-WR infection at 10^7 PFU and 66% died after infection of 10^6 PFU VV-WR. Similar to rVV I-TAC infected C57BL/6 mice, VV-WR infected mice were able to resolve infection when challenged with 10^5 and 10^4 PFU.

Athymic Swiss nude mice have a defective immune system with immunity dependent primarily on NK cells activity. Figure 5.4 shows the mortality rate for Swiss nude mice infected with different doses of VV-WR or rVV I-TAC. Some Swiss nude mice were able to fully resolve infection of rVV I-TAC after infection with 10^4 and 10^5 PFU, but not with 10^6 or 10^7 PFU, while all mice succumbed to infection and died at all doses of VV-WR. This suggests that I-TAC can also attract NK cells that can reduce vaccinia viremia.

The mean time to death (MTD, in days) for both strains of mice, at varying doses of VV-WR and rVV I-TAC, are shown in table 5.1. The number of mice that died versus total numbers used is shown in brackets. The MTD was dose dependent in both strains of mice with the MTD being delayed in rVV I-TAC mice compared to VV-WR infected mice. The MTD was also found to be mouse strain dependent as the Swiss nude mice succumbed to infection and died earlier and with lower doses of virus than C57BL/6 mice.

5.3.4 Histological studies in virus infected mice

Lungs, liver and ovaries were isolated and processed from groups of 6-8 week old C57BL/6 and athymic Swiss nude mice infected with either VV-WR or rVV I-TAC (1 x 10^6 PFU i.p) on days 0, 3, 6, and 9 (for C57BL/6 only) and histological slides were prepared, fixed and stained with hematoxylin and eosin. Figure 5.5 shows a representative sample of lungs on days 0, 3, 6 and 9 mice infected with either VV-WR or rVV I-TAC. There was a significant increased in the inflammation and mononuclear cellular infiltration observed in C57BL/6 mice infected with rVV I-TAC than those with VV-WR in all tissues analyzed. Day 6 showed the highest level of cellular infiltration compared to days 0, 3 and 9.

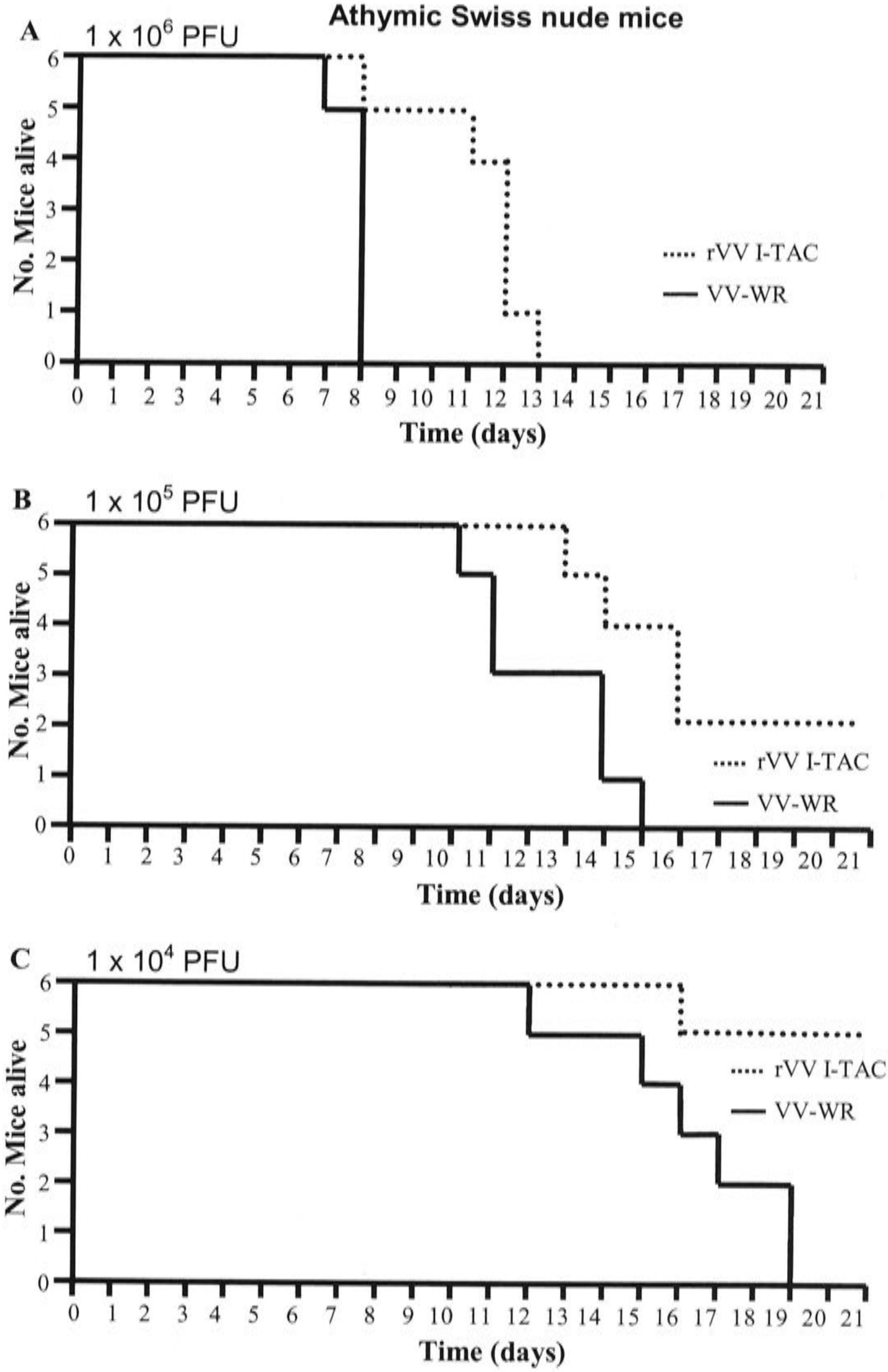


Figure 5.4: Mortality studies. Groups of 6 athymic Swiss nude mice were challenged i.v. with either VV-WR or rVV I-TAC at varying doses with their time of death being recorded. Doses used were 1 x 10^7 PFU (not shown), 1 x 10^6 PFU (A), 1 x 10^5 PFU (B) and 1 x 10^4 PFU (C). There was no significant difference in the survival rates of mice challenged with 1 x 10^7 PFU, with all dying by day 8. Solid lines (——) represent mice infected with VV-WR, whereas dashed lines (·····) represent mice infected with rVV I-TAC.

Table 5.1: Mean time to death (days) for both athymic Swiss nude mice and C57BL/6 mice at varying doses of VV-WR and rVV I-TAC. The number of mice that died versus total numbers used is shown in brackets.

	Swiss nude mice		C57BL/6 mice	
Viral dosage (PFU)	VV-WR	rVV I-TAC	VV-WR	rVV I-TAC
1×10^7	6.5 (6/6)	7.3 (6/6)	6.3 (6/6)	8.3 (3/6)
1×10^6	8.8 (6/6)	11.5 (6/6)	10.5 (4/6)	- (0/6)
1×10^5	12.5 (6/6)	14.8 (4/6)	- (0/6)	- (0/6)
1×10^4	15.0 (4/6)	16.0 (1/6)	- (0/6)	- (0/6)

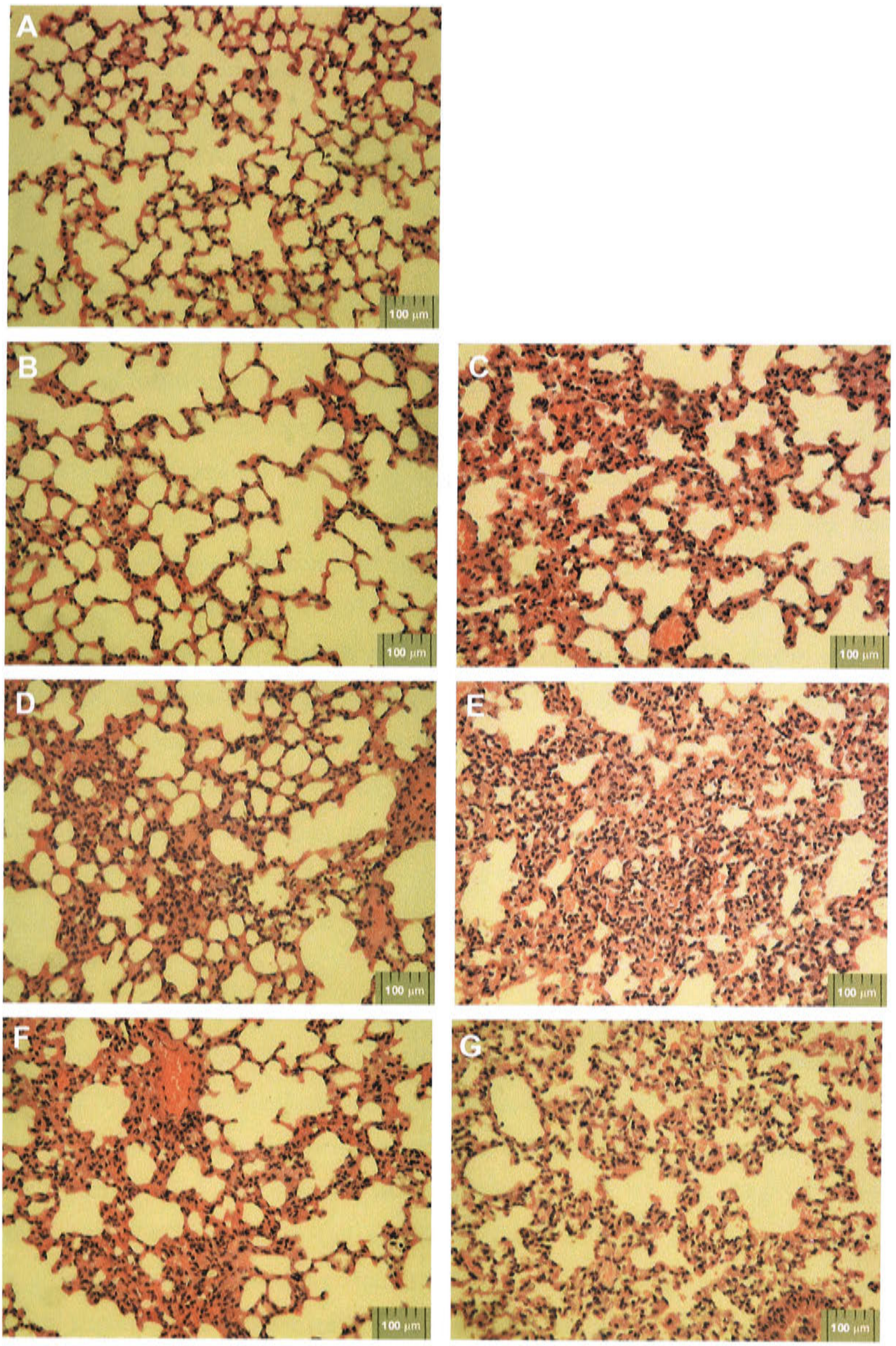


Figure 5.5: Histology of C57BL/6 mouse lungs. Uninfected (A), VV-WR infected mice on days 3 (B), 6 (D), 9 (F) and rVV I-TAC infected mice on days 3 (C), 6 (E) and 9 (G).

Figure 5.6 shows a representative sample of lungs on days 0, 3 and 6 from naïve Swiss nude mice infected with either VV-WR or rVV I-TAC. Like the C57BL/6 mice, increased inflammation and mononuclear cellular infiltration is observed in mice infected with rVV I-TAC than those infected with VV-WR in all tissues analyzed. Day six shows the highest level of infiltration. Swiss nude mice infected with VV-WR succumbed to infection before samples could be collected at day 9. In both strains of mice similar results to lung tissue was observed in both ovaries and liver.

5.3.5 Footpad infiltration in VV infected C57BL/6 mice

To examine the ability of murine I-TAC to recruit cells, two methods of measuring inflammation was examined. Footpads of naïve mice were injected with 1 x 10^7 PFU of either VV-WR or rVV I-TAC to measure the relative rate of swelling as the first measure of inflammation. Mice injected with VV-WR had significantly larger footpads than VV I-TAC challenged mice, although the rate of recovery was similar (Fig 5.7A). This result indicated that expression of I-TAC, elicited by VV, might reduce inflammation. Mice preimmunized (with 1 x 10^5 PFU of VV-WR) were also subjected to the same footpad analysis as a second measure of inflammation (Fig 5.7B). VV preimmune mice injected with VV-WR had significantly larger footpads than rVV I-TAC challenged mice. In addition, the rate of swelling reduction was faster in rVV I-TAC mice than VV-WR infected mice (Fig 5.7B). Overall, the initial increase in footpad size was greater and the rate of swelling reduction slower, in naïve mice compared to VV preimmune mice.

The data suggested that I-TAC has a role in reducing inflammation. Histology of footpads 24 hrs post viral challenge gave an indication of cellular recruitment to the site of infection. There was a significant increase in cellular infiltrate in the naïve mice infected with rVV I-TAC compared to those infected with VV-WR (Fig 5.8). This suggests that I-TAC expression may recruit NK and T cells that respond to infection more efficiently, thereby reducing overall inflammation. In the VV preimmune mice however, slightly more cellular infiltration is observed in mice infected with VV-WR than those injected with rVV I-TAC (Fig 5.8). I-TAC may be recruiting memory T cells to the site of infection again resulting in a faster clearance of virus.

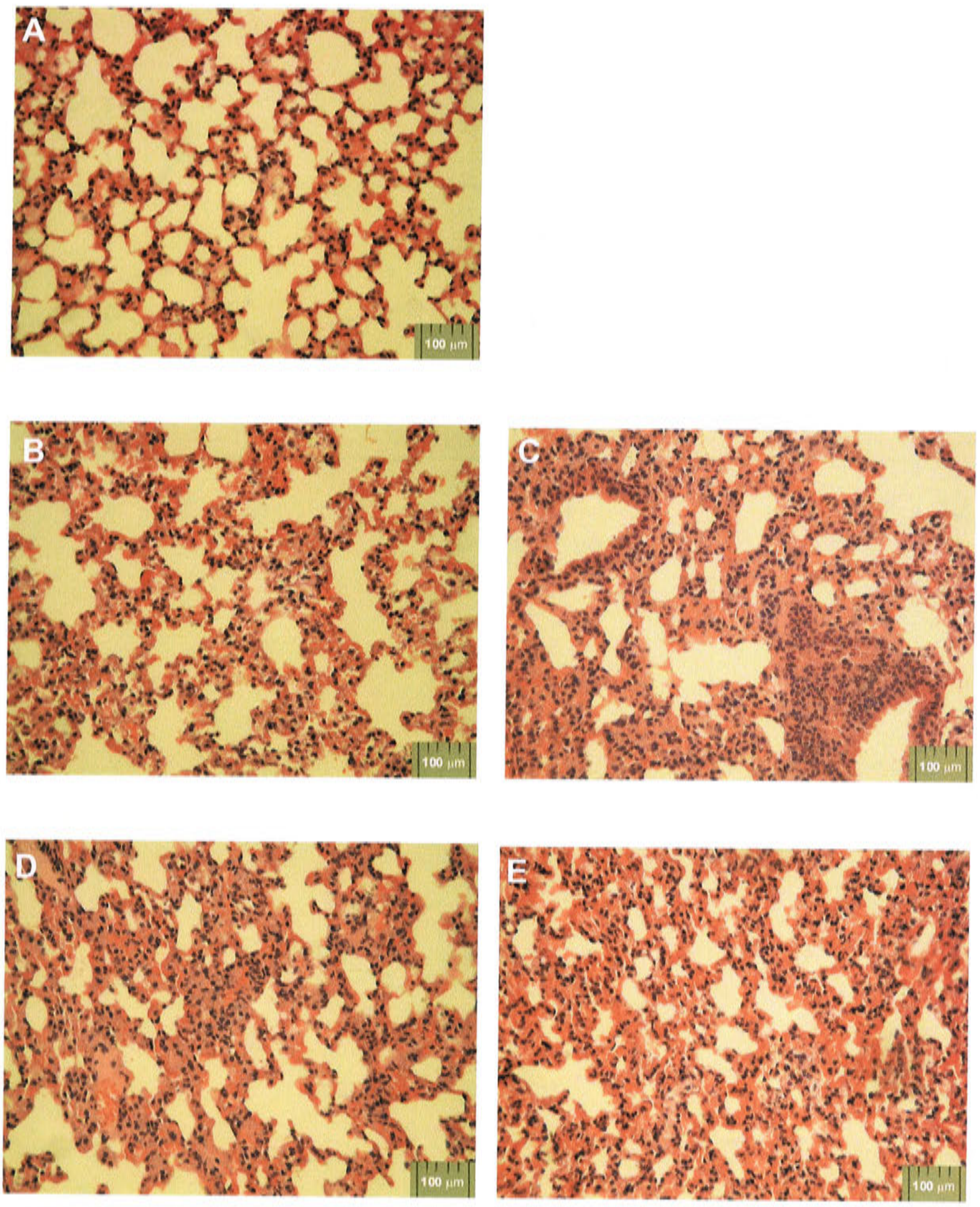
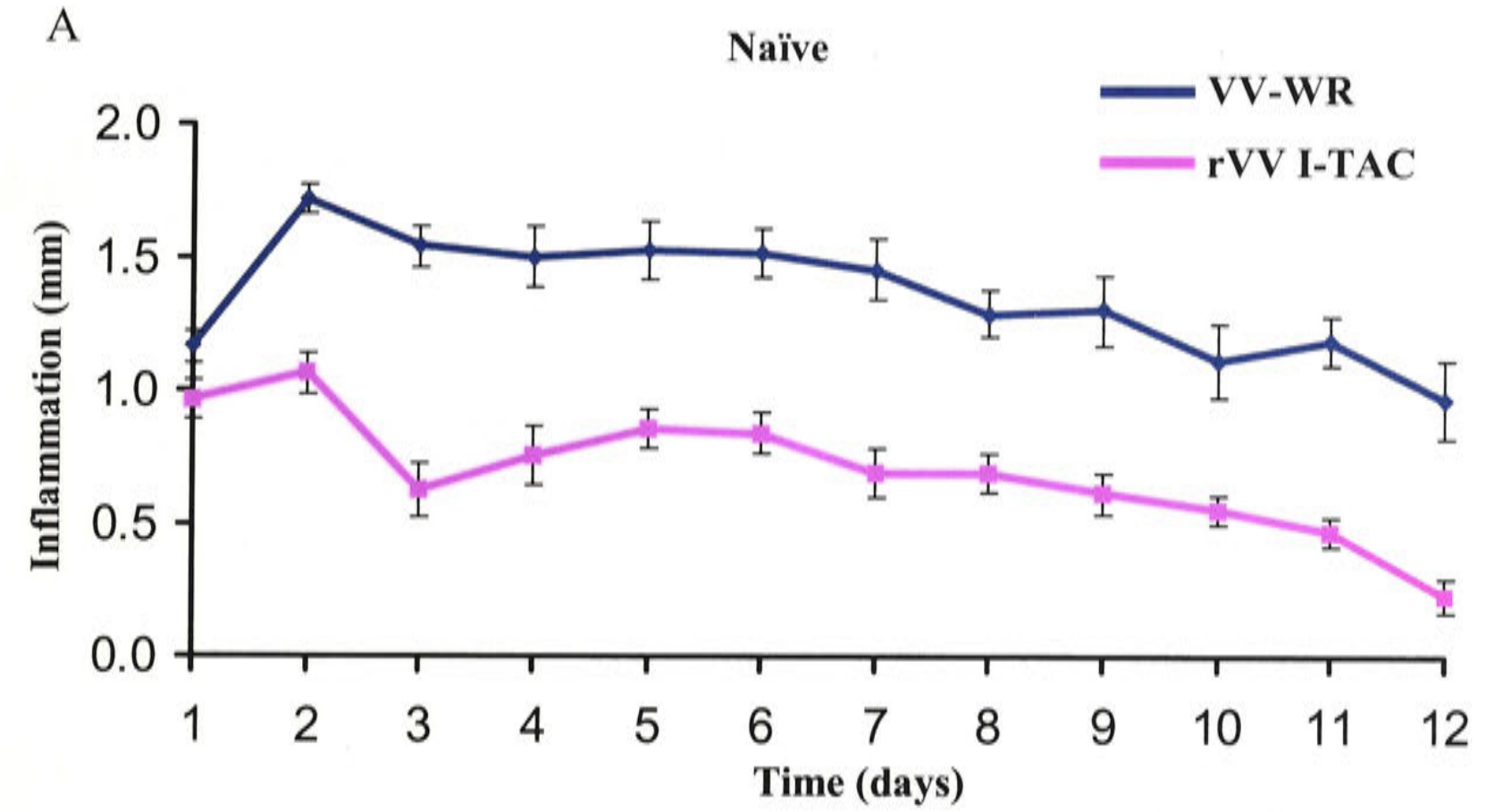


Figure 5.6: Histology of lungs from athymic Swiss nude mice. Uninfected (A), Day 3 post infection with VV-WR (B) or rVV I-TAC (C), and day 6 post infection with VV-WR (D) or rVV I-TAC (E). Swiss nude mice infected with VV-WR succumbed to infection before samples could be collected at day 9 post infection.



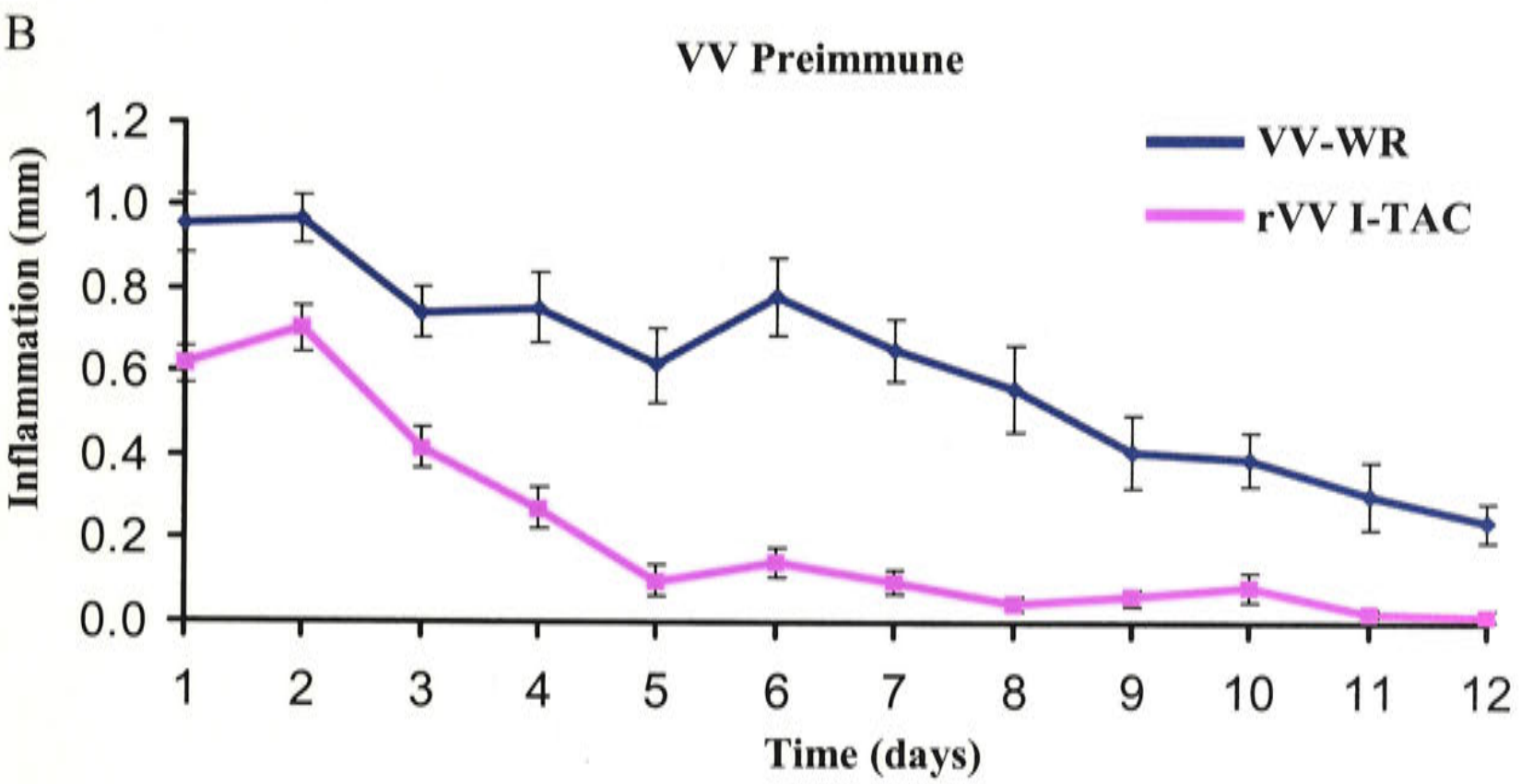


Figure 5.7: Footpad inflammation. Groups of 4 naïve C57BL/6 mice (A) or VV preimmune (with $1x10^5$ PFU of VV-WR) C57BL/6 mice (B) were challenged with VV-WR or rVV I-TAC ($1x10^7$ PFU) injected into each hind footpad. Footpad thickness was measured daily. Basal footpad thickness is standardized to zero swelling for each mouse. Standard errors of the mean are shown, all results except for day 1 for naïve mice (A) are significantly different ($p < 0.005$; student's t-test).

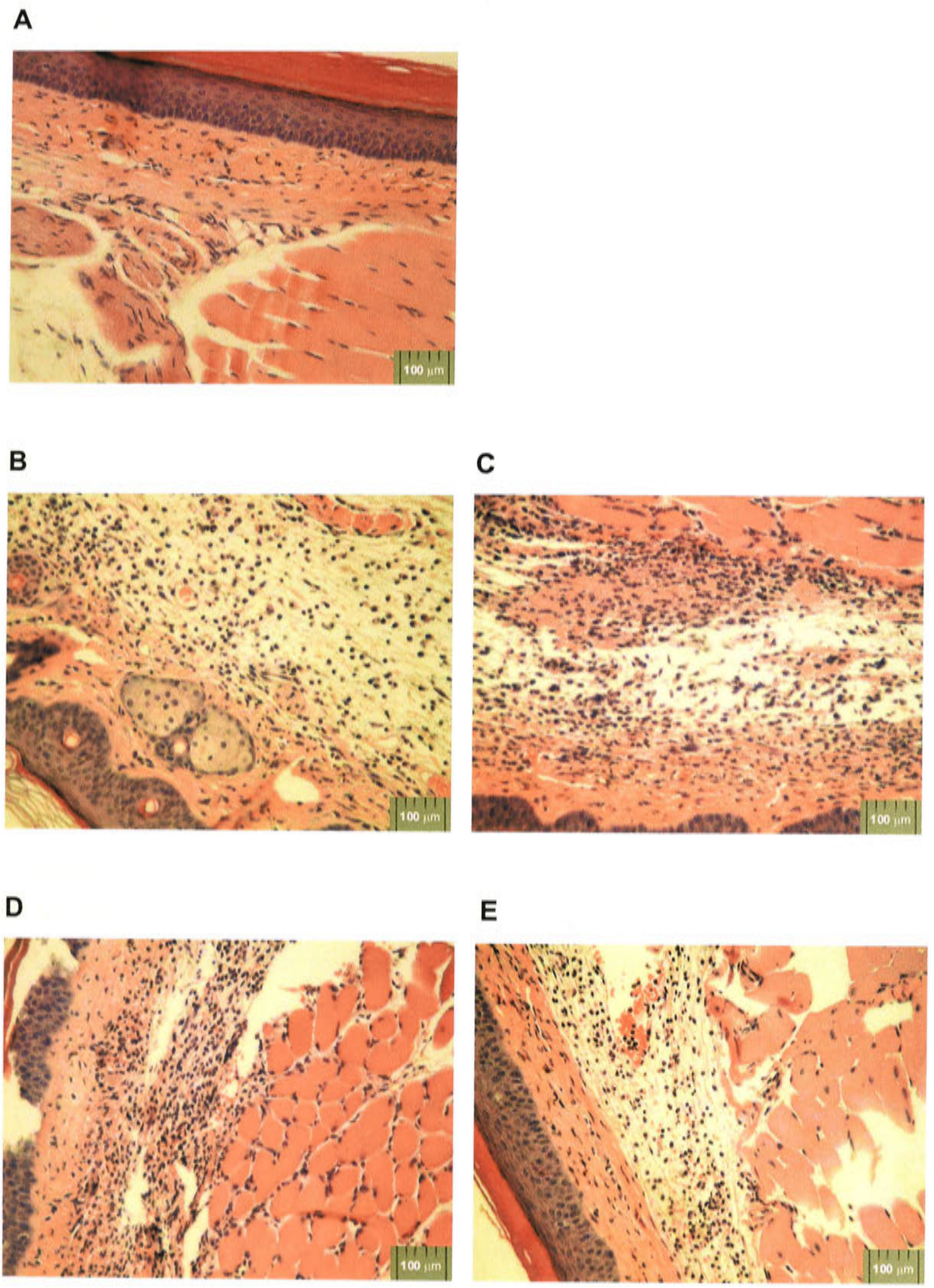


Figure 5.8: Histology of footpads from C57BL/6 mice 24 hrs post challenge. Representative samples of uninfected (A), naïve mice infected with VV-WR (B) or rVV I-TAC (C), or VV preimmune mice infected with VV-WR (D) or VV I-TAC (E) are shown.

5.4 Discussion

Murine I-TAC is expressed during various viral infections such as RSV and Influenza A (chapter 4) and therefore this molecule may play an important role in viral clearance. Previous studies have shown that the IFNs and IFN-inducible chemokines Mig and Crg-2 are important for the control of viral infection (Dalton *et al.*, 1993; Huang *et al.*, 1993; Muller *et al.*, 1994; Mahalingam *et al.*, 1999). Surprisingly, murine I-TAC was not detected during a VV infection (chapter 4), which may be due to the virus having a mechanism that inhibits the expression of chemokines that are detrimental to the survival of the virus.

A recombinant VV encoding I-TAC was able to express the chemokine as a functional protein and recruit Con A activated splenocytes. Viral titres in C57BL/6 mice were lower in rVV I-TAC challenged mice than those in mice challenged with control virus. The lower level of virus demonstrates that I-TAC either enhances viral clearance or restricts viral replication *in vivo*, or a combination of both. During mortality studies the control virus (VV-WR) proved lethal at high doses in C57BL/6 mice and at moderate to low doses in athymic Swiss nude mice whereas there was marked attenuation in those mice challenged with rVV I-TAC. I-TAC expressed from rVV did not provide complete protection at high doses, however the mean time to death for each respective VV dose was delayed in all groups of mice tested. I-TAC therefore provided some mechanism to slow virus replication at low doses and provide some protection at higher doses but not sufficient to clear the virus.

Enhanced mononuclear cell infiltration was observed in histological slides of liver, lungs and ovaries in both C57BL/6 mice and athymic Swiss nude mice infected with rVV I-TAC at several time points during infection. In addition to this, in Swiss nude mice, rVV attenuation compared to the control virus is not as marked as in C57BL/6 mice. The cellular infiltration in both strains of mice correlates with the time course of infection with peak infiltration occurring around the peak of infection. By comparing these results with previous studies examining rVV Mig and rVV Crg-2, it is likely that the cells recruited by I-TAC were T cells and NK cells in the C57BL/6 mice and predominantly NK cells in the athymic Swiss nude mice (Mahalingam *et al.*, 1999; Mahalingam and Karupiah, 2000a).

Measuring footpad thickness in naïve mice after viral challenge showed lower peak swelling in mice challenge with rVV I-TAC than challenged with the control virus. While at first glance this appears surprising, this corresponds well with the notion that I-TAC enhances the recruitment of specific cells, primarily NK and Th1 cells to control the viral infection reducing viremia and the overall number of immune cells infiltrating the footpad. While this was not directly examined the experiments carried out using VV preimmunized mice suggest the observation is consistent with this interpretation.

The effect of preimmunizing mice with low doses of VV-WR before challenge significantly reduced footpad swelling compared to naïve mice probably by recruitment of specific T and B cells. Expression of I-TAC via rVV I-TAC in such mice resulted in a further reduction of swelling in mice post viral challenge. In addition to this, the rate at which this swelling decreased was faster in rVV I-TAC challenged mice than mice challenged with wild type virus. The recruitment of primed memory T cells would explain why, in VV preimmune mice, infiltration and inflammation is less in rVV I-TAC infected mice compared to VV-WR infected mice. This subsequently would lead to rVV I-TAC challenged mice resolving infection more efficiently with less overall infiltrate and inflammation and presumably reduced viremia. These results however, are for one virus model and it would be interesting to see if similar results can be obtained in different pathogen infections, especially in disease models where dependence on cell-mediated clearance mechanisms are not as pronounced.

CHAPTER 6.

FINAL DISCUSSION

CHAPTER 6. FINAL DISCUSSION

This thesis explores the characteristics of the murine chemokine, I-TAC. These were examined by a number of methods, including sequence analysis, *in vitro* and *in vivo* expression studies, as well as functional studies during different disease models. The ability of murine I-TAC to modify immune responses was also analyzed. Together these experiments provide a broad characterisation of the role that I-TAC has in the immune system.

A complete cDNA clone of the murine chemokine, I-TAC was isolated from an IFN-γ stimulated dendritic cell line using a cDNA subtraction method. The cDNA clone was sequenced and confirmed subsequently by published data and is now also referred to as murine CXCL11 according with new international nomenclature (Meyer *et al.*, 2000; Widney *et al.*, 2000). Murine I-TAC is a non-ELR CXC chemokine that is 67% homologous to HuI-TAC and ~35% homologous to Crg-2 and MuMig respectively. The open reading frame is 100 amino acids long with a predicted 21 amino acid leader sequence, which coincides with the leader sequence of HuI-TAC. If cleaved at this point murine I-TAC would be a 79 aa residue mature protein with a molecular mass of 9112 Da (Meyer *et al.*, 2000; Widney *et al.*, 2000). In addition, murine I-TAC maps to chromosome 5 at position 5E3, which is close to the positions of MuMig and Crg-2 (Meyer *et al.*, 2000; Widney *et al.*, 2000). Moreover murine I-TAC contains four exons with intron/exon boundaries at similar positions to human I-TAC (Meyer *et al.*, 2000). Together, these data confirm a strong mouse/human homology and as a result the two proteins would probably have the same, or very similar, functions *in vivo*.

As indicated earlier, murine I-TAC binds to the chemokine receptor, CXCR3 and induces a dose dependent calcium signal (Meyer *et al.*, 2001). As a result murine I-TAC, like human I-TAC, is likely to attract activated CXCR3 expressing NK and T lymphocytes, predominantly of the Th1 phenotype. Calcium mobilization assays have demonstrated that calcium signals were obtained using 10 nM murine I-TAC whereas higher concentrations (300 nM) of MuMig and Crg-2 were required to obtain the same signal (Meyer *et al.*, 2001). Like its human counterpart it has been demonstrated that murine I-TAC is a more potent chemokine than either MuMig or Crg-2 (Meyer *et al.*,

2001). The relative potency of murine I-TAC in the published data above was demonstrated by chemotaxis assays using CXCR3 transfected B cells where optimum migration rates required 3 nM of murine I-TAC as compared to 30 nM of Crg-2 and 100 nM MuMig (Meyer *et al.*, 2001).

Although the stability of I-TAC *in vivo* is unknown, the similarities of potency, rate of migration, as well as receptor binding between the murine and human chemokine homologues allows functional chemokine gradient models to be speculated. There is a potency hierarchy between the three chemokines with I-TAC being the most potent followed by IP-10/Crg-2, then Mig (Cole *et al.*, 1998; Cox *et al.*, 2001; Meyer *et al.*, 2001). It is possible that a chemokine gradient is set up with Mig being released locally, with IP-10/Crg-2 having a wider dispersal area. As much lower concentrations of I-TAC are required for effective migration this potent chemokine could be widely dispersed throughout the body and away from the site of infection. NK and T cells would firstly be exposed to I-TAC and subsequently follow the increasing chemokine concentration to IP-10/Crg-2 then to Mig to fight infection.

There are however several pieces of data suggesting that the above model is not the case, firstly human I-TAC is able to displace IP-10 or HuMig bound to the CXCR3 receptor which also occurs in a concentration dependent manner whereas HuMig nor IP-10 can not completely displace HuI-TAC (Cole *et al.*, 1998). Secondly, it has been shown that during a *Toxoplasma gondii* or VV infection Crg-2 expression was co-localized with a respective infection site whereas MuMig was expressed more systemically (Amichay *et al.*, 1996). Thirdly, peak murine I-TAC expression occurred later than peak Crg-2 expression in both IFN-γ and LPS stimulated RAW264.7 macrophage cells and in lungs from mice given LPS (Widney *et al.*, 2000). This differential expression has also been observed in the MTHC-D2 cells (Banyer *et al.*, 2002 submitted, appendix 1c). Interestingly, CD40 activation enhanced levels of I-TAC expression in these cells (chapter 3) whereas Crg-2 expression was reduced (Banyer *et al.*, 2002 submitted, appendix 1c). Together, these features suggest a different chemokine gradient model where the timing and dispersal areas of the chemokines would be important. Mig may be dispersed early and widely followed by IP-10/Crg-2. The expression of I-TAC may occur later and locally and displace the other chemokines

from CXCR3. If this were the case, I-TAC would be the last chemokine to bind to the CXCR3 receptor. This chemokine receptor would then be desensitized, a rapid process rendering the cell unresponsive to stimulation through that receptor (Chuang *et al.*, 1996). The receptor is then internalized for degradation or recycling which subsequently re-establishes chemokine responsiveness (Solari *et al.*, 1997; Aragay *et al.*, 1998; Signoret *et al.*, 1998). In a recent study it was demonstrated that CXCR3, expressed on T cells, was rapidly internalized when exposed to IFN-γ activated human saphenous vein endothelial cells (HSVEC) (Sauty *et al.*, 2001). Importantly it was shown that I-TAC was the primary chemokine responsible for internalization even though it was secreted at lower levels than both IP-10 and Mig (Sauty *et al.*, 2001). The stability of the IFN-inducible chemokines is a determining factor for which model is operating, for example while I-TAC may be the most potent chemokine, it may also be the most unstable, supporting the second gradient model.

Different binding regions to the CXCR3 receptor and with varied affinities, suggests that the three IFN-inducible chemokines not only establish a gradient for cells to migrate follow, but may have distinct functions *in vivo* (Cox *et al.*, 2001). Indeed, expression levels of these three chemokines differ between cells (Amichay *et al.*, 1996; Mach *et al.*, 1999). During the development of atherosclerotic lesions, four major cells are involved including endothelial cells, smooth muscle cells, macrophages and lymphocytes (Ross, 1999). Examining chemokine expression from these cells within atherosclerotic plaques, a differential chemokine expression pattern was found. Endothelial cells, smooth muscle cells and macrophages all expressed IP-10, whereas only endothelial cells and macrophages express HuI-TAC (Mach *et al.*, 1999). Different expression levels of the chemokines would enable a chemokine gradient for the migration of T lymphocytes and NK cells to be initiated as described above. Differential expression between cell types within a lesion however, suggests different functions within the immune response, though this remains to be elucidated.

Examining the signalling pathway responsible for I-TAC induction can give an insight into the type of immune response that involves I-TAC and hence an insight into the chemokine's function. The DC line and knockout mouse data indicated I-TAC is primarily induced by IFN-γ via the transcription factor IRF-1 (chapter 3). Published

findings also support that IFN-γ is the major cytokine required for I-TAC expression (Meyer *et al.*, 2000; Widney *et al.*, 2000; Meyer *et al.*, 2001). As a result, other signalling transcription factors crucial for IFN-γ signalling are also implicated in murine I-TAC expression including the Janus kinases, STAT1 and NF-κB (reviewed in Stark *et al.*, 1998; Schindler, 1999; Taniguchi *et al.*, 2001). While IFN-γ initiates a number of different anti-pathogen mechanisms, it is possible that when there is low or absent IFN-γ signalling, such as in IFN-γR KO mice, the reduced amount of I-TAC and probably IP-10 and Mig results in increased susceptibility to infection. This would result in immune cells having reduced migration efficiency to the site of infection. Mice lacking IFN-γR are far more susceptible to infection by *Listeria monocytogenes* and VV despite apparently normal cytotoxic and T helper cell responses is one example of this (Huang *et al.*, 1993). This explanation becomes more complex when autoimmunity is examined. For example, the EAE data in chapter four demonstrated that the IFN-γR knockout mice, which had no detectable I-TAC expression were far more susceptible to disease thereby raising questions regarding the kinetics of autoreactive T cell recruitment into the CNS.

The experiments presented here (chapter 2) also clearly demonstrated that IL-4 suppressed murine I-TAC expression. This is not surprising considering that the Th1 cytokines, such as IL-12 and IFN-γ, can down regulate Th2 cytokines including IL-4, and vice versa (reviewed in Rook *et al.*, 1994; Ivashkiv, 1995; Yasukawa *et al.*, 2000). IL-4 can inhibit STAT1 via STAT6 activation, which subsequently would inhibit I-TAC expression (Larner *et al.*, 1993). Therefore one of the ways IL-4 could inhibit IFN-γ immune responses may include the down regulation of I-TAC via the inhibition of the IFN-γ signalling pathway.

Others have also demonstrated that IFN-α/β can weakly induce both human and murine I-TAC whereas the knockout mouse studies in chapter 3 did not confirm this (Rani *et al.*, 1996; Widney *et al.*, 2000; Meyer *et al.*, 2001). Low levels of IFN-α/β can down regulate IRF-1 via IRF-2 activation and probably subsequent I-TAC expression as seen in Con A activated splenocytes (Watanabe and Kawade, 1988; Noronha *et al.*, 1993; Mamane *et al.*, 1999). However, stimulation with higher concentrations of these cytokines may in fact induce low levels of I-TAC. Hence it appears that IFN-α and

IFN-β may act to either increase or decrease I-TAC expression depending on the relative amount of these two cytokines, and subsequent level of IRF-1 activation or inhibition.

Synergy between TNF-α and IFN-γ to enhance the expression of murine I-TAC has been observed (Widney *et al.*, 2000). The degree at which this occurs however appears to be dependent on initial levels of IFN-γ induced I-TAC, for example, strong synergistic effects are observed in Swiss 3T3 cells where initial IFN-γ induction of murine I-TAC is low (Widney *et al.*, 2000). However, in the cell line or Con A culture studies carried out here (chapter 2), the levels of murine I-TAC were initially relatively high, no evidence of synergy between IFN-γ and TNF-α was observed.

The addition of anti-CD40 Ab to MTHC-D2 cells showed that CD40 alone can upregulate expression of murine I-TAC and that the CD40 and IFN-γ pathways can synergize to enhance this expression. These results are consistent with activation of the NF-κB transcription factor through independent binding sites by IFN-γ, TNF-α and CD40 individually as well as in a synergistic manner (Ohmori and Hamilton, 1995; Baldwin, 1996; van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). It is likely that this effect is cell type and/or stimulus specific as synergy between IFN-γ and TNF-α was not observed in the induction of I-TAC in the DC lines (chapter 2) whereas in other cells synergy has been observed (Ohmori and Hamilton, 1995; Huang *et al.*, 2000; Widney *et al.*, 2000). In addition to this, enhanced I-TAC expression was not detected in the JAWS II cell line following CD40 activation, which may however, be due to the immortalization process of JAWS II DC line.

The studies carried out here suggest that murine I-TAC is expressed from murine progenitor DCs exposed to IFN-γ *in vivo*. Bone marrow derived DCs as well as other cell lines such as RAW264.7 macrophages, Swiss 3T3 fibroblasts and J744A.1 cells have all subsequently been shown to express murine I-TAC after IFN-γ stimulation. The expression levels however, differ considerably depending on the cell type which may be reflective of their function *in vivo* (Meyer *et al.*, 2000; Widney *et al.*, 2000; Meyer *et al.*, 2001). For example, RAW264.7 macrophages can induce high levels of murine I-

TAC whereas relatively low levels are observed in IFN-γ stimulated Swiss 3T3 fibroblasts (Widney *et al.*, 2000).

As I-TAC, Mig and IP-10/Crg-2 are expressed from DCs and other APCs and recruit $CXCR3^+$ NK and T lymphocytes it is likely that I-TAC and the other IFN-inducible chemokines are important in the interactions between the innate and adaptive immune systems. Indeed, many chemokines including the IFN-inducible chemokines have been detected during viral infections aiding the CMI response to clear infection. Expression of murine I-TAC around peak infection indicates that I-TAC has a role in enhancing viral clearance such as during RSV and influenza infections, probably by recruiting $CXCR3^+$ expressing NK and Th1 cells.

One interesting finding from this work was that I-TAC expression was not detectable during a VV infection whereas others have shown that Mig and Crg-2 were present (Mahalingam *et al.*, 2000; Mahalingam and Karupiah, 2000a). As these three chemokines have different expression patterns *in vivo*, it is likely that they have different regulatory factors controlling signalling pathways. In fact it has previously been shown that Crg-2 can be upregulated by both the IFN-γ and IFN-α/β signalling pathways whereas Mig is stimulated by IFN-γ only (Amichay *et al.*, 1996). VV induced inhibition of I-TAC but not Crg-2 or Mig expression may occur at some point during the IFN-γ signalling pathway, possibly through altering activation states or expression levels of the STAT1 or NF-κB transcription factors (Bowie *et al.*, 2000; Gil *et al.*, 2001; Najarro *et al.*, 2001; Oie and Pickup, 2001). VV and other poxviruses such as ectromelia and variola (smallpox) are very successful viruses due to their ability to avoid the immune system (reviewed in Smith *et al.*, 1997; Mahalingam and Karupiah, 2000b; Moss and Shisler, 2001). Through constant evolution, they have developed several mechanisms to inhibit expression of antiviral cytokines, chemokines and their receptors (reviewed in Smith *et al.*, 1997; Mahalingam and Karupiah, 2000b; Moss and Shisler, 2001). Inhibition of I-TAC, the most potent known IFN-inducible chemokine would therefore be a logical target for poxviruses. Indeed over-expression of I-TAC through the use of a recombinant VV significantly attenuates viral growth and efficacy demonstrating how valuable I-TAC is in viral clearance. Poxviruses belonging to the same genera tend to have similar immune evasion genes. Although VV is relatively

harmless to humans, and is in fact commonly used as a vaccine against smallpox, it is also closely related to smallpox as well as other orthopoxviruses including cowpox, ectromelia and monkeypox. As a result, each of the orthopoxviruses share many conserved immune evasion genes (reviewed in Smith *et al.*, 1997; Moss and Shisler, 2001). It is likely then that smallpox virus may also have the ability to inhibit I-TAC expression, aiding to its success as a human disease.

The ability of I-TAC to recruit NK and Th1 cells does not automatically mean that I-TAC is beneficial in all diseases. Others have speculated that I-TAC can exacerbate neuroinflammation and autoimmunity since it can recruit activated T cells (Cole *et al.*, 1998; Luo *et al.*, 1998). Given that antibodies to a functional domain of CXCR3 protect mice from EAE, murine I-TAC may act to enhance neuroinflammatory processes (Arimilli *et al.*, 2000). The expression of murine I-TAC during peak EAE disease may mean that I-TAC has an integral role in autoreactive T cell recruitment. Many studies have also demonstrated that T cell recruitment is important during the rejection process of transplants and indeed, inhibition of T cell migration has been shown to inhibit or at least delay the onset of the rejection process (Russell *et al.*, 1996; Chandraker *et al.*, 1998; Melter *et al.*, 1999; Hancock *et al.*, 2000). Although a trend of murine I-TAC expression was not clear during the transplantation grafts carried out in this study, it is likely that this was due to the models utilized. Murine I-TAC as well as Crg-2 and MuMig have been shown to be upregulated during a cardiac allograft rejection model (Meyer *et al.*, 2001). All of these studies have confirmed that I-TAC is expressed during immune responses in various disease situations. The outcome of I-TAC activity whether it is beneficial, such as in viral infections, or detrimental, such as in autoimmune diseases, most likely depends on the disease in question. I-TAC expression during viral infections probably supports viral clearance by attracting NK and Th1 cells to the site of infection, whereas during EAE, I-TAC may exacerbate disease by recruiting autoreactive T cells into the CNS.

The data acquired in this study has enabled the formulation of a two-part model, showing how I-TAC may interact with the rest of the immune system especially during a disease where the CMI response is required, such as during an influenza viral infection. At the site of infection, chemokines that are expressed by infected cells under inflammatory conditions, including IL-8, MIP-1α, RANTES, MCP-1 and MCP-4,

recruit immature DCs and other innate immune cells such as NK cells and macrophages (Cyster, 1999; Sallusto *et al.*, 2000; Marquez and Martinez, 2001). While such migrating DCs are beginning to mature as well as being exposed to antigen they are also exposed to cytokines such as IFN-γ, TNF-α, IL-1 and bacterial or viral products such as LPS or dsRNA which initiate the expression of chemokines including I-TAC, Mig and IP-10/Crg-2. The expression of the IFN-inducible chemokines occurs relatively quickly (within 6 hours) after DCs have been stimulated with IFN-γ, as has been shown in chapter 2 and by others (Banyer *et al.*, 2002 submitted, appendix 1c; Meyer *et al.*, 2001). Circulating memory T cells would migrate directly to the site of infection and an adaptive immune response would then be initiated against the antigen (Fig 6.1). For this to be possible, memory T cells must express the CXCR3 receptor, and indeed this has been shown by others previously (Qin *et al.*, 1998; Sallusto *et al.*, 1998). Such a response would occur when the host has previously been exposed to the antigen, as occurs in vaccinated hosts, for example the preimmunized mice in the footpad experiment carried out in chapter 5.

The second part of the model outlines the process that may occur if the animal has not previously been exposed to antigen (Fig 6.2). DCs would migrate to the site of infection as described above, however, once DCs become fully mature a complete alteration in chemokine receptor expression also occurs. The receptors CXCR4, CCR4 and CCR7 are all up regulated while the initial receptors expressed at the infection site are all down regulated. This enables the DC to then migrate to the lymph node to activate the adaptive immune response, all the while secreting I-TAC, Mig and IP-10/Crg-2 (Cyster, 1999; Sallusto *et al.*, 2000; Marquez and Martinez, 2001). Along with chemokine receptor expression switching, a significant change in chemokine expression from DCs also alters during later stages of maturation. The chemokines expressed also differ depending on the stimuli that the DC received while maturing as well as the effector cells that are required for activation, i.e. a Th1 or Th2 phenotype (Sallusto *et al.*, 1999; Sallusto and Lanzavecchia, 2000). For the activation of a Th1, cell mediated immune response expression of I-TAC, Mig and IP-10/Crg-2 are also upregulated during this time of DC maturation (Sallusto *et al.*, 1999; Sallusto and Lanzavecchia, 2000; Meyer *et al.*, 2001). The expression of I-TAC may also be enhanced further by CD40-CD40L interactions during the antigen presentation process.

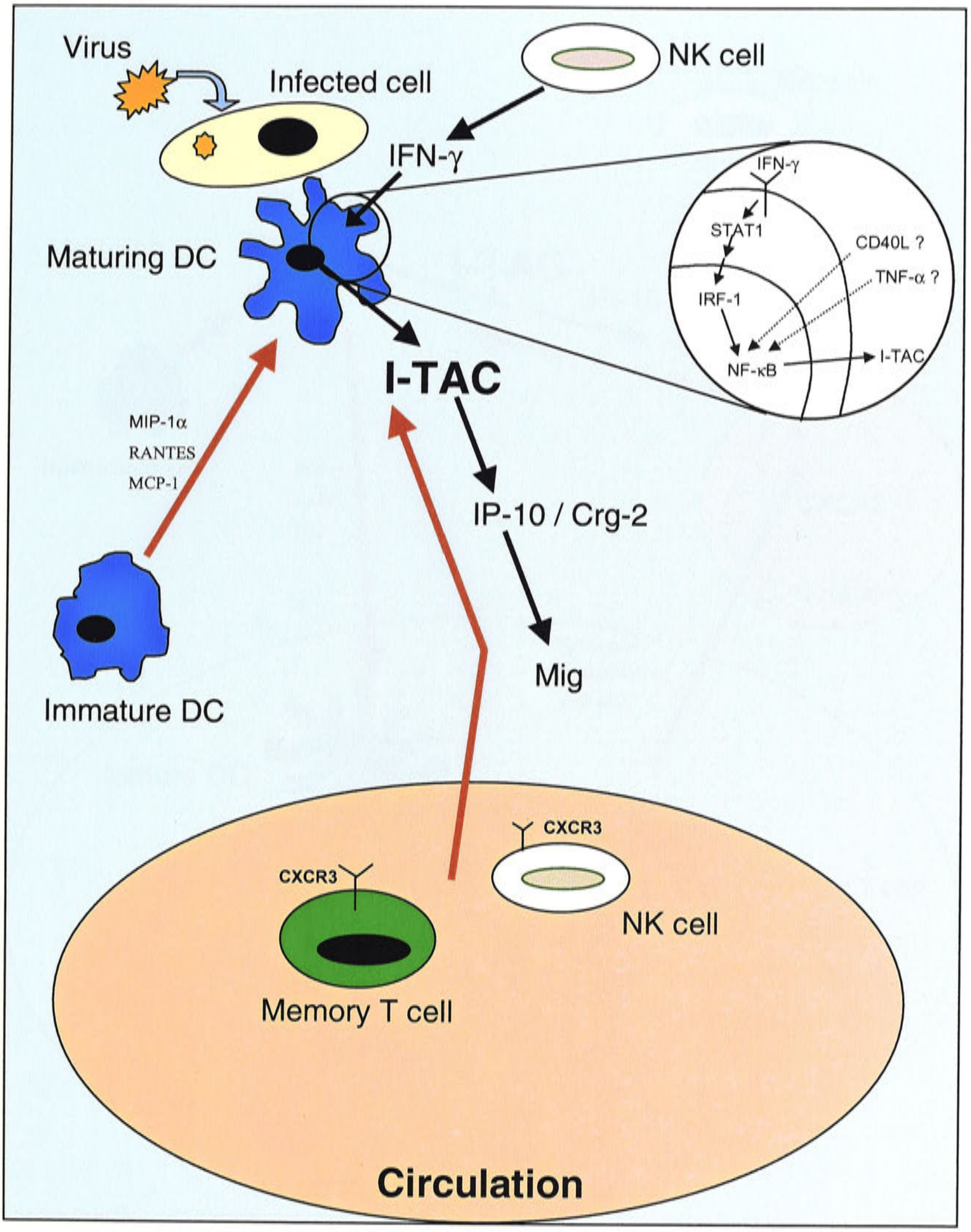


Figure 6.1: Schematic diagram showing how murine I-TAC might interact in the immune system during a viral infection (part 1). Red arrows show migration pathways, black arrows show cytokine or chemokine expression.

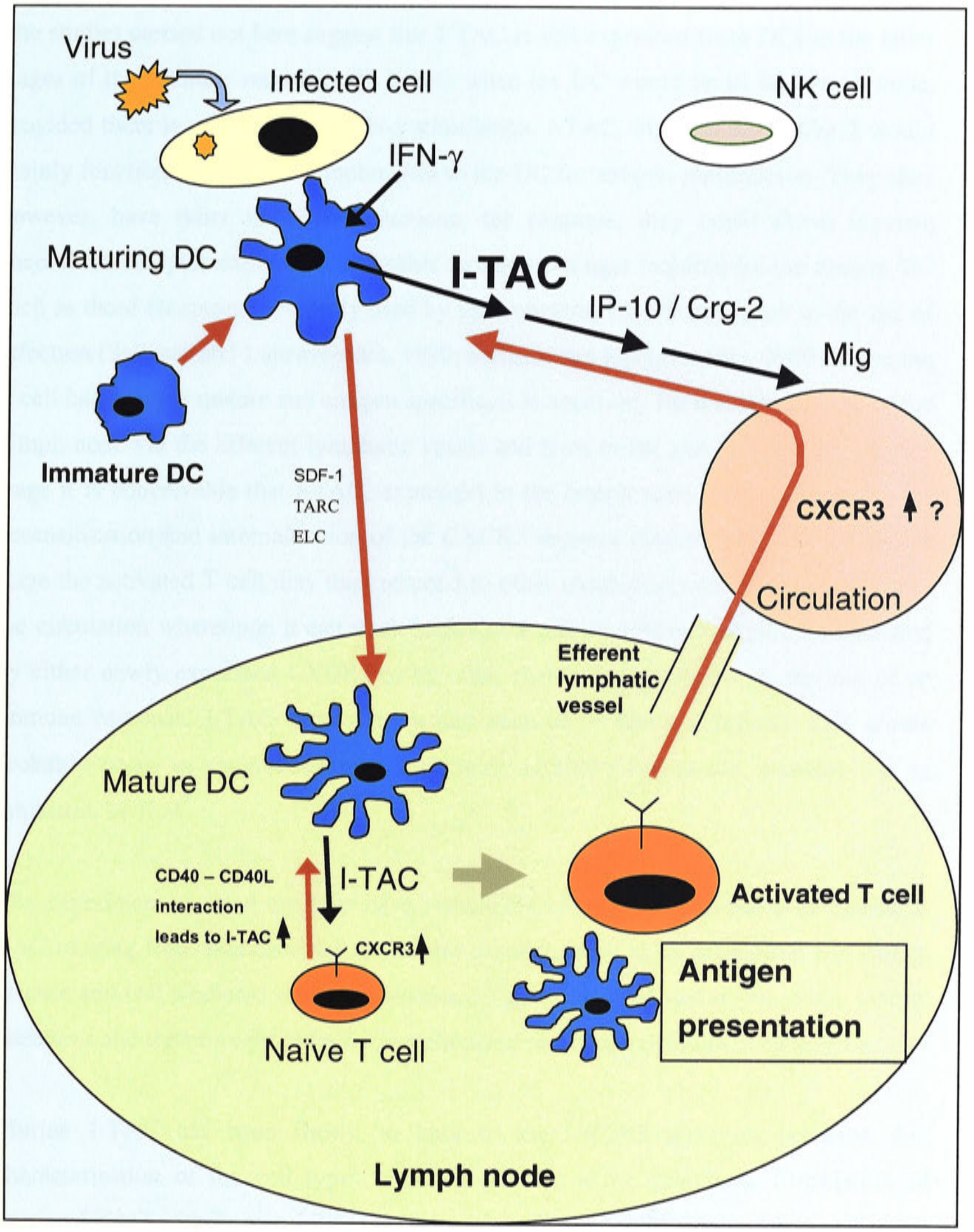


Figure 6.2: Part 2 of how murine I-TAC might interact in the immune system during a viral infection. Red arrows show migration pathways, black arrows show cytokine or chemokine expression.

The studies carried out here suggest that I-TAC is still expressed from DCs in the latter stages of the immune response (48 hours) when the DC would be in the lymph node, provided there is still sufficient IFN-γ stimulation. I-TAC, Mig and IP-10/Crg-2 would mainly function to recruit T lymphocytes to the DC for antigen presentation. They may however, have other important functions, for example, they could down regulate chemokine receptor expression and other factors no longer required by the mature DC such as those receptors previously used by the immature DC for migration to the site of infection (Sallusto and Lanzavecchia, 1999; Sallusto and Lanzavecchia, 2000). Once the T cell has become mature and antigen specific, it is necessary for it to migrate out of the lymph node via the efferent lymphatic vessel and back to the site of infection. At this stage it is conceivable that I-TAC, expressed in the lymph node is responsible for the desensitization and internalization of the CXCR3 receptor (Sauty *et al.*, 2001). At this stage the activated T cell may then respond to other chemokines and migrate back into the circulation whereupon it can track back to the site of infection, a process mediated by either newly expressed CXCR3 or by other chemokine gradients. At the end of an immune response, I-TAC expression would need to be down regulated. This would probably occur in conjunction with a decrease in IFN-γ expression, possibly due to inhibition by IL-4.

The experiments carried out here have provided a broad characterization of murine I-TAC ranging from sequence and molecular characteristics to its expression and role in disease and cell mediated immune responses. The model proposed above raises several questions and therefore provides a foundation and direction for future investigation.

Murine I-TAC has been shown to bind to the CXCR3 receptor, however, full characterisation of the cell types recruited remains to be examined. Correlation of murine I-TAC with human I-TAC and the other IFN-inducible chemokines will most likely demonstrate that this chemokine recruits T cells and NK cells. The range of cells recruited in mice however, needs to be investigated. In addition to this, further elucidating the signalling pathways of the IFN-inducible chemokines in detail will aid the understanding of the different chemokine expression patterns necessary for, and in response to, various immune stimuli. This in turn may enable specific chemokine targeting for vaccines and therapeutics, in such diseases as multiple sclerosis. Further

understanding of the role that I-TAC has in immune responses may be addressed by creating an I-TAC knockout mouse. Compensation by the other IFN-inducible chemokines however, may occur so that the immunological effect of I-TAC might be masked. The exact mechanism whereby VV inhibits I-TAC expression is not clear however, once the signalling pathway for I-TAC induction has been thoroughly elucidated, the factor(s) that VV inhibits may be more easily identified.

Autoimmune disease progression has been inhibited by blocking the CXCR3 receptor, presumably blocking the function of multiple chemokines (Arimilli *et al.*, 2000). Being the most potent IFN-inducible chemokine, it is possible that developing vaccines to block I-TAC alone may produce a more direct and specific therapy. In preliminary studies however, passive EAE in the rat was not inhibited by treatment of recipients with anti-rat ITAC (David Willenborg, personal communication). Co-expressing I-TAC in conjunction with a vaccine or therapeutic agents may aid the establishment of cell mediated immunity. One possibility could be to generate a cancer antigen presenting DC, capable of also secreting I-TAC to enhance T cell recruitment and subsequent priming and activation against a specific tumour.

The generation of polyclonal antibodies to I-TAC, using the baculovirus protein expression system for example, could allow for further characterisation of this chemokine. Antibodies could be used for several assays including blocking I-TAC receptor binding studies, I-TAC secretion and tracking analyses. Blocking I-TAC *in vivo* in diseases, other than EAE, for example during viral diseases may further aid the understanding of immune responses that are normally induced. Having antibodies to the three IFN-inducible chemokines may also allow for the elucidation of their individual functions to be more thoroughly characterised.

In conclusion, the work presented in this thesis explores the characteristics of the murine chemokine, I-TAC. The results of these studies not only highlight the properties exhibited by murine I-TAC but gives a valuable insight into the attributes that human I-TAC and the other IFN-inducible chemokines, Mig and IP-10 have, especially with respect to their role in the immune system. With this knowledge and the rate of research into chemokines generally, the use of chemokines in therapeutic agents may become feasible in the not too distant future.

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APPENDIX

APPENDIX 1A

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Cytokines in innate and adaptive immunity

Key words:
adaptive immunity; chemokine; cytokine; dendritic cells; innate immunity

Abstract: Cytokines and chemokines are hormone-like messengers which act to regulate the development and expression of the broad array of immune responses that are mounted against a variety of pathogens. As such, they are critical determinants of the types of cells which will regulate and participate in innate and adaptive immune responses, they may act both in highly localised environments but also in a systemic manner, and they may, themselves, directly mediate antimicrobial effector activities. In this article, we will outline current concepts of the activities of cytokines and chemokines in the immune response and discuss the various cell types, including dendritic cells and other antigen-presenting cells, T cells and B cells, which both produce and respond to these potent regulatory molecules.

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Host defense mechanisms are numerous and range from relatively primitive and constitutively expressed, non-specific defenses to sophisticated adaptive mechanisms that are specifically induced in response to foreign antigen (invading pathogens). Although the role of innate immunity was originally considered to be a process for defense of the host early in infection, it is now clear that there is an important reciprocal relationship between innate and adaptive immune responses. Cytokines and other mediators play an essential role in this process and, indeed, may ultimately determine the type of effector response that is generated towards a pathogen. These factors are first produced at sites of infection following interactions of pathogens and danger signals from damaged tissues. This process requires recognition of invariant molecular structures common to a variety of pathogens by innate immune cells; these are called pathogen-associated molecular patterns. Well known examples of microbial stimulators of innate immune responses include lipopolysaccharides and teichoic acids shared by a variety of bacteria and unmethylated CpG motifs found in microbial but not mammalian DNA. Host organisms have evolved cellular receptors with broad specificities that can recognise these molecules and detect infectious

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microbes. The immediate result of this recognition is the production of chemokines and cytokines that not only activate the innate cell population but also drive the adaptive immune response down different pathways of differentiation. Cytokines may be grouped into two broad categories; those that act to stimulate Th1 type immune responses, which mainly protect against intracellular viral, bacterial, fungal and protozoan pathogens, (including IL-12 and IFN-γ) and those that stimulate Th2 responses involved in protection against extra cellular infections such as helminths (including IL-4, IL-5, IL-6, IL-10 and IL-13). The cytokine microenvironment also influences innate cell populations, including macrophages and natural killer (NK) cells, and particularly stimulating the differentiation of dendritic cells (DC) with distinct immunoregulatory properties which may promote Th1- or Th2-type adaptive immune responses. The profile of chemokines expressed by innate immune cells may also influence the phenotype of the immune response by directing the homing of different T cell subsets to sites of infection. This has the effect of amplifying the immune response, a process which is also mediated by the effector cytokines produced by local T cells which act in a feedback loop on the innate immune cells. To ensure that inflammatory cell-mediated immune responses do not cause too much damage to the host, the immune system has inbuilt protective mechanisms. Those with which we are most familiar include the reciprocal cross-regulatory effects of Th1 and Th2-type effector cytokines, such as IFN-γ and IL-4. However, Th2-type cytokines may also have strong down regulatory effects on the Th1-promoting properties of DC. Thus, innate and adaptive immune responses are intimately linked and controlled by sets of molecules and receptors that act to generate the most effective form of immunity suitable for protection against each given pathogen. In addition, improved understanding of how these molecules may influence the immune response has facilitated their use as immunological adjuvants in a variety of vaccination strategies. In this article, we will outline current concepts of cytokine regulation of the development and expression of antimicrobial immune responses.

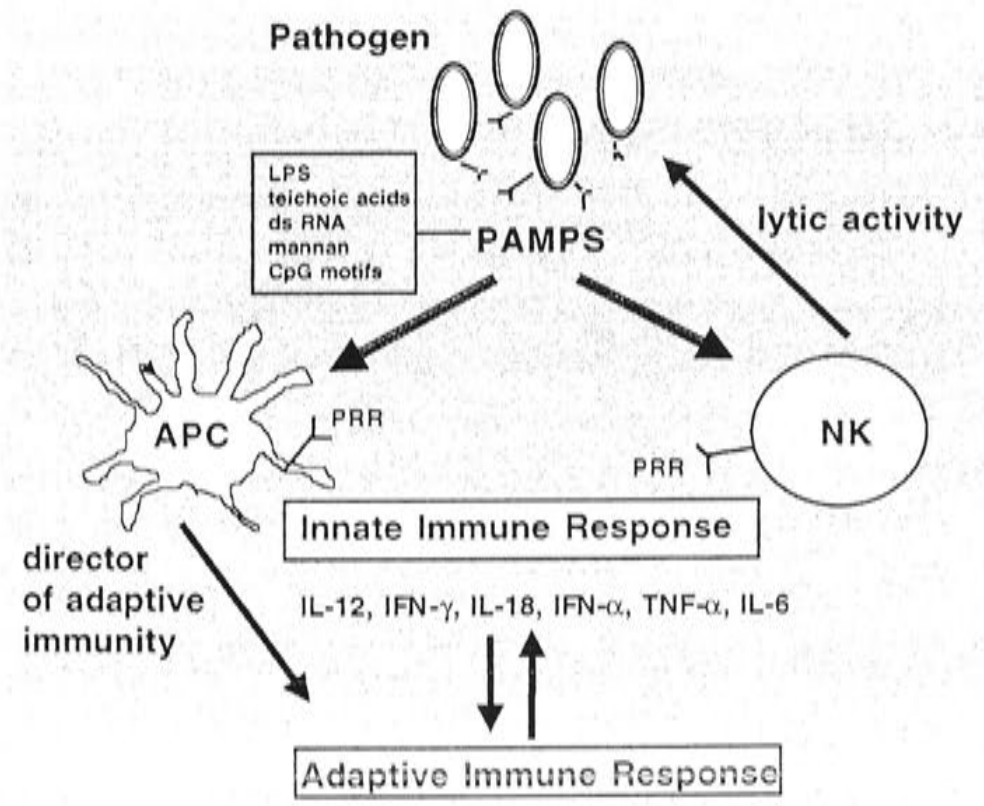

Fig. 1. **Immunological determination of self and non-self.** Invading microorganisms expressing pathogen-associated molecular patterns (PAMPs) are recognised by innate immune cells (including macrophages, dendritic cells and NK cells) via pattern recognition receptors (PRR). This process signals to innate cells the presence of foreign organisms and activates the secretion of a myriad of effector cytokines which play a variety of roles in directing the immune response.

Initiation of innate immune responses

An efficient immune system must achieve, during development, the ability to distinguish self from non-self. The enormous variability, molecular heterogeneity and rapid mutational rates of invading pathogens adds to the difficulty of this task. There are, however, characteristic structural features that are shared by many pathogens and which may be recognised by the innate immune system. These are termed pathogen-associated molecular patterns (PAMPs). PAMPs include lipopolysaccharides and teichoic acids which are shared by all gram-positive and gram-negative bacterial DNA, double stranded RNA which is characteristic of RNA viruses, and yeast cell walls which contain mannan (1, 2). The conserved nature of PAMPs is illustrated by the bactericidal effect of penicillin, which targets PAMPs and inhibits peptidoglycan synthesis in bacteria (2). Since PAMPS are not produced in vertebrates, host cells have developed non-clonal, broad specificity receptors that can recognise a wide range of pathogens. These are termed pattern recognition receptors (PRR) which are encoded in the germline and expressed on cells that will encounter a pathogen early in infection, e.g. epithelial cells and effector cells of the innate immune system.

As shown in Fig. 1, engagement of PRR with PAMPs create signals which can be divided into several pathways: (i) those that induce opsonization of bacteria for phagocytosis and/or the activation of the lectin pathways of complement, (ii) those that promote uptake of pathogens by phagocytes and DC, and (iii) those that induce effector functions which initiate the expression of inflammatory cytokines and initiate adaptive immunity (2).

PAMP/PRR interactions are important for stimulating cellular secretion of effector cytokines (Fig. 1). The types of effector cytokines which are produced by cells of the innate immune system, and particularly DC, are influenced by the types of antigen which

bind particular PRR on innate cells. For example the mannose receptor, which is expressed on macrophages and DC, induces expression of several cytokines, including IL-1, IL-6, GM-CSF, TNF-α, IL-12 and IL-18. Bacterial cell wall engagement of another PRR, the FcγR, down regulates secretion of IL-12 from DC, thereby favouring the development of a Th2-type adaptive immune response (3). However, virus-binding PRR induce expression of Type 1 IFNs (α and β), factors which potently increase expression of class I MHC molecules on DC and also increase the efficiency of presentation of viral peptides to cytotoxic T cells (4).

Effector cytokines are, in turn, important for regulating expression of PRR, e.g. IFN-γ and other factors that down regulate transcription of the mannose receptor and endotoxic activity in activated macrophages and the up regulation of this activity by IL-4. Interestingly, IFN-γ can enhance mannose receptor-mediated phagocytosis and killing of the yeast, *Candida albicans*, indicating that endocytic activity mediated through ligation of.this receptor is independent from mannose receptor phagocytic activity (5). Whilst it is believed that PAMPs are the major determinant factor in innate immune recognition, there are probably additional mechanisms involved, including the 'recognition of altered self' and the 'absence of self'. In the case of viral DNA pathogens, where all of the components of the virus are synthesized and assembled internally in the host cells, it may be assumed that there are no recognisable PAMPs. How the immune system recognises such pathogens is unclear and requires further study.

Unmethylated CpG motifs from prokaryotic pathogens have been targeted as likely candidates for binding to PRR (6). Recent evidence indicates that the CpG receptor is intracellular, however the mechanism of action and downstream signalling pathways remain to be elucidated (6, 7). Activation of NFκB and the mitogen-activated protein kinase (MAPK) pathway have been associated with CpG processing (8–10). A variety of cell types may be activated by CpG DNA directly, including macrophages, DC, NK cells and B cells and are stimulated to release a variety of cytokines, predominantly Th1 cell-inducing factors, such as IL-12 and IL-18 (11). Increased understanding of the mechanism of CpG DNA recognition by the immune system now provides researchers with an opportunity to incorporate these motifs into vaccine preparations in order to further enhance their efficacy.

Importantly, antigen binding of PRR, CpG activity, phagocytosis and stimulation by effector cytokines all promote the maturation of DC and their migration from peripheral tissues via afferent lymphatics or blood to lymphoid organs. In their more mature state, DC upregulate expression of the co-stimulatory molecules CD80 and CD86 and also clonal antigen-presenting class I and II MHC antigens. At this point, co-stimulatory molecules may communicate to adaptive immune cells the presence of recognition sequences from infectious agents, whilst the clonal MHC receptors communicate the nature of the pathogen which is to be targeted. More recently, communication of a third signal, directing the nature of the adaptive immune response, has been assigned to DC, however the nature of this signal is unknown (12).

Induction of Th cell differentiation by innate immune cell cytokine production

It is now clear that different sub-populations of Th cells develop from the same precursor T cell, an immature CD4-bearing T lymphocyte which secretes IL-2 upon stimulation by antigen which is often termed a Th0 cell (13). Several factors are now known to be important in determining whether this cell will ultimately take on Th1- or Th2-type characteristics. The cytokine milieu at the point of initiation of the immune response is a key determinant of the commitment of precursor cells to a particular developmental pathway (14). For example, viral and bacterial pathogens, in part through their possession of PAMPS binding to PRR and, through recognition of CpG DNA motifs, usually stimulate innate immune cells to produce factors which promote the development of Th1 cells. Thus, DC and macrophages commonly secrete large amounts of IL-12, and subsequently NK cells produce IFN-γ, the effects of which are to drive Th1 cell differentiation from the Th0 precursor cell. This results in the development of a Th1-type immune response, the effects of which, in combination with the innate response itself, are to assist in the eradication of microbial pathogens. Conversely, the early production of IL-4 by a variety of innate cell types directs Th2 cell development, although the relative importance of each of these sources remains to be clarified (13, 15).

The predominant role of IL-12 as a determinant of Th1 cell development is now well-established (16, 17). IL-12 signals primarily through the transcription factors, signal transducer and activator of transcription-3 (Stat3) and Stat4 (18, 19), although the recent discovery of T-bet provides clear evidence for further important lineage-specific transcription factors in Th1 cell development (20). T-bet, which belongs to the T-box family of transcription factors, is rapidly and selectively induced in developing Th1 cells (but not Th2 cells), upregulating, in turn, the expression of IFN-γ. This factor is inducible by IL-12, a process enhanced by IL-18 and diverts naïve T cells into the Th1 differentiation pathway. T-bet may even convert committed Th2 cells into Th1-type cells, at least partly via repression of IL-4 and IL-5 gene expression (20).

Functional receptors for IL-12 are expressed on recently acti-

vated, committed naïve T cells and Th1 cells, but are lost during Th2 cell differentiation. The unresponsiveness of Th2 cells to IL-12 may thus be explained by down regulation of the IL-12 receptor β2 chain (IL-12Rβ2) by IL-4. Conversely, IFN-γ acts to upregulate IL-12Rβ2 expression by Th1 cells and counteracts the activity of IL-4 in this respect (21, 22). Two further cytokines, IL-1α and IL-18, may play important roles as co-factors in the induction of Th1 cell development and IFN-γ production (23). IL-18 (previously known as IFN-γ-inducing factor, IGIF) is structurally related to IL-1; indeed its receptor is termed IL-1 receptor-related protein (IL-1Rrp) (24). IL-18, like IL-12, is expressed from activated macrophages, DC, keratinocytes, astrocytes, osteoblasts, microglia and Kupffer cells, and induces IFN-γ expression by splenocytes, B cells and Th1 cells (25–27). However, unlike IL-12, IL-18 does not directly activate Stat4 in Th1 cells, and does not appear to act independently, rather it synergises with IL-12 in the production of IFN-γ and, therefore, in the development of Th1 cell-mediated immune responses (25, 26).

Just as IL-12 is the key factor in Th1 cell lineage commitment, IL-4 appears to play a similar role in initiating Th2 cell development. Indeed, the influence of IL-4 on naïve Th cells appears to be dominant over that of IL-12, such that threshold levels of IL-4 at the point of induction of an immune response will lead to Th2 cell development and upregulated IL-4 production (13, 28). IL-4 signals through the transcription factor Stat6 (29) to upregulate expression of IL-4, IL-5 and IL-13, although Stat activation is a transient event and is unlikely, of itself, to determine Th cell phenotype development as this is a process which takes several days (14, 30). Additional lineage-specific transcription factors also appear to be required; c-Maf increases IL-4 expression (31), whilst GATA-3 induces expression of several Th2 factors in both developing and committed T cells and may inhibit IFN-γ secretion (32, 33). The source of IL-4 early in the immune response which is important for Th2 cell differentiation remains controversial. Non-T cells, including mast cells, basophils and eosinophils are prime candidates (13), as are CD4+ and CD4−CD8− T cells of the NK1+ subset (15) and LACK (*Leishmania* homologue of receptors for activated kinase C)-specific CD4+ T cells expressing Vβ4 and Vβ8 TCR (34). Recently, it has also been suggested that certain pathogens may trigger IL-4 secretion from DC themselves (35). In particular, a key role for NK1+ cells in Th2 cell development was suggested by their capacity to produce large amounts of IL-4 immediately following activation (15). However, mice deficient in CD1, the selecting antigen for NK1+ cells, were able to mount Th2-type responses in the absence of this cell subset (36). Whilst IL-4 is clearly a critical mediator of Th2 differentiation, the mechanisms underlying its activity are yet to be fully understood.

Clearly, cells of the innate immune system, in addition to their roles in mediating early, non-specific immune responses, play a variety of key functions in initiating the development of different lineages of Th cells and in the ultimate expression of immunity. Whilst macrophages, NK cells, mast cells, basophils and eosinophils are all important players in these respects, it is now evident that the activity of DC is vital for the co-ordinated development of the adaptive immune response.

Dendritic cells and regulation of the immune response

Discrete sets of DC are strategically positioned to perform the important functions of antigen sampling and triggering of T and B lymphocyte development. The origins and inter-relationships of different DC subtypes is unresolved, however the highly plastic nature of these cells indicates that differences in tissue location and antigenic and cytokine stimuli are major determinants of DC phenotype. They are in fact, a product of their microenvironment and recent studies have clearly demonstrated the determinant effects of Th1 and Th2-type cytokine expression on DC and their subsequent capacity to stimulate either Th1 or Th2-type adaptive immune responses (37) (Figs. 2 and 3).

There are conflicting theories on the origins of different DC lineages, however a consensus has now been established that distinct DC populations exist in different tissues and perform different immunoregulatory activities. These include DC in the periphery, which efficiently take up and process antigens and then migrate to lymphoid tissues where they interact with T and B cells. In these lymphoid tissues, further DC subtypes interact with T cells, probably mediating the maintenance of tolerance to self antigens. Peripheral DC are known as myeloid-related DC, whilst resident lymphoid tissue DC are thought to be lymphoid in origin (12, 38, 39).

The types of DC which develop and the nature of the adaptive immune response which is ultimately expressed correlate strongly with the levels of bioactive IL-12 produced by these cells. A number of studies have established a link between DC subtype, IL-12 secretion and the polarization of Th1 and Th2-type adaptive immunity (Fig. 2). In the mouse, CD8α+, CD11c+ lymphoid-related DC secrete IL-12 and promote Th1-type responses, whereas CD8α−, CD11c+ myeloid DC, which do not secrete IL-12, promote Th2-type responses (40). Interestingly, the opposite association is apparent in humans, where lymphoid-related DC do not secrete IL-12 and promote Th2-type responses, whilst myeloid DC secrete high levels of IL-12 and promote Th1-type responses (41). To complicate matters further, additional DC types have been described, including the $CD8\alpha^{-}CD4^{-}$ murine DC, which secrete low levels of IL-12 (38), and

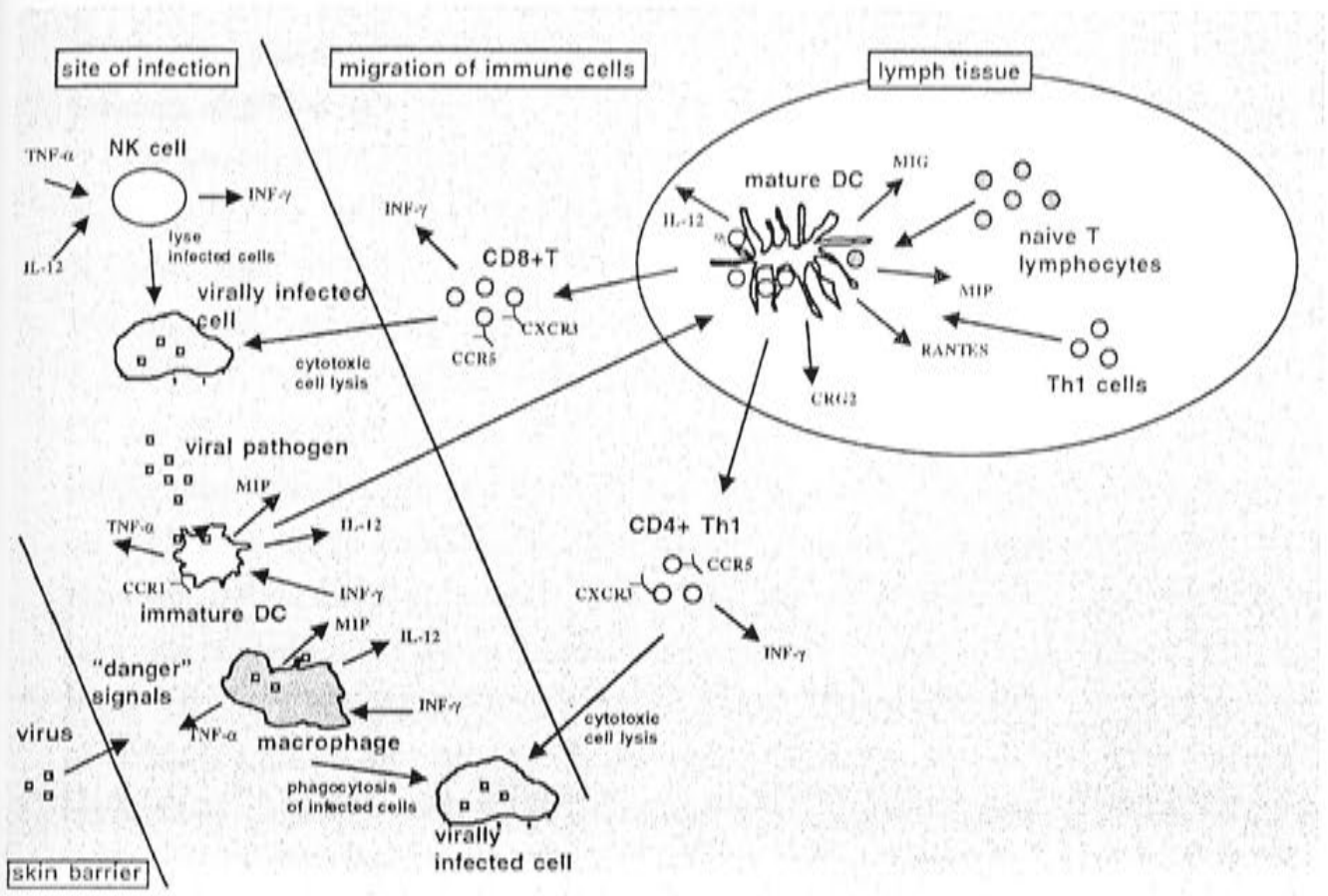

Fig. 2. **The influence of dendritic cells on the development of cell-mediated immunity.** Immediately following infection by intracellular pathogens, a combination of danger signals from surrounding tissues, effector cytokines and the pathogen itself, all trigger immature dendritic cells to undergo maturation. This process leads to antigen processing, increased surface expression of MHC molecules and co-stimulatory molecules, secretion of chemoattractants (which act to recruit other leukocytes), and secretion of bioactive IL-12. The dendritic cell then migrates to lymphoid tissues and secretes further chemoattractants which recruit activated, memory Th1 and naive T cells. These T cells are activated, in the presence of co-stimulatory molecules, by the antigen, which is presented to the T cell receptor as peptides associated with MHC molecules. Activated T cells secrete IFN-γ and an assortment of chemokines, and also express chemokine receptors, which facilitate their sequential migration to sites of infection. Here, they exert antigen-specific effector activity against infected cells and also act on immature dendritic cells to further promote cell-mediated immunity.

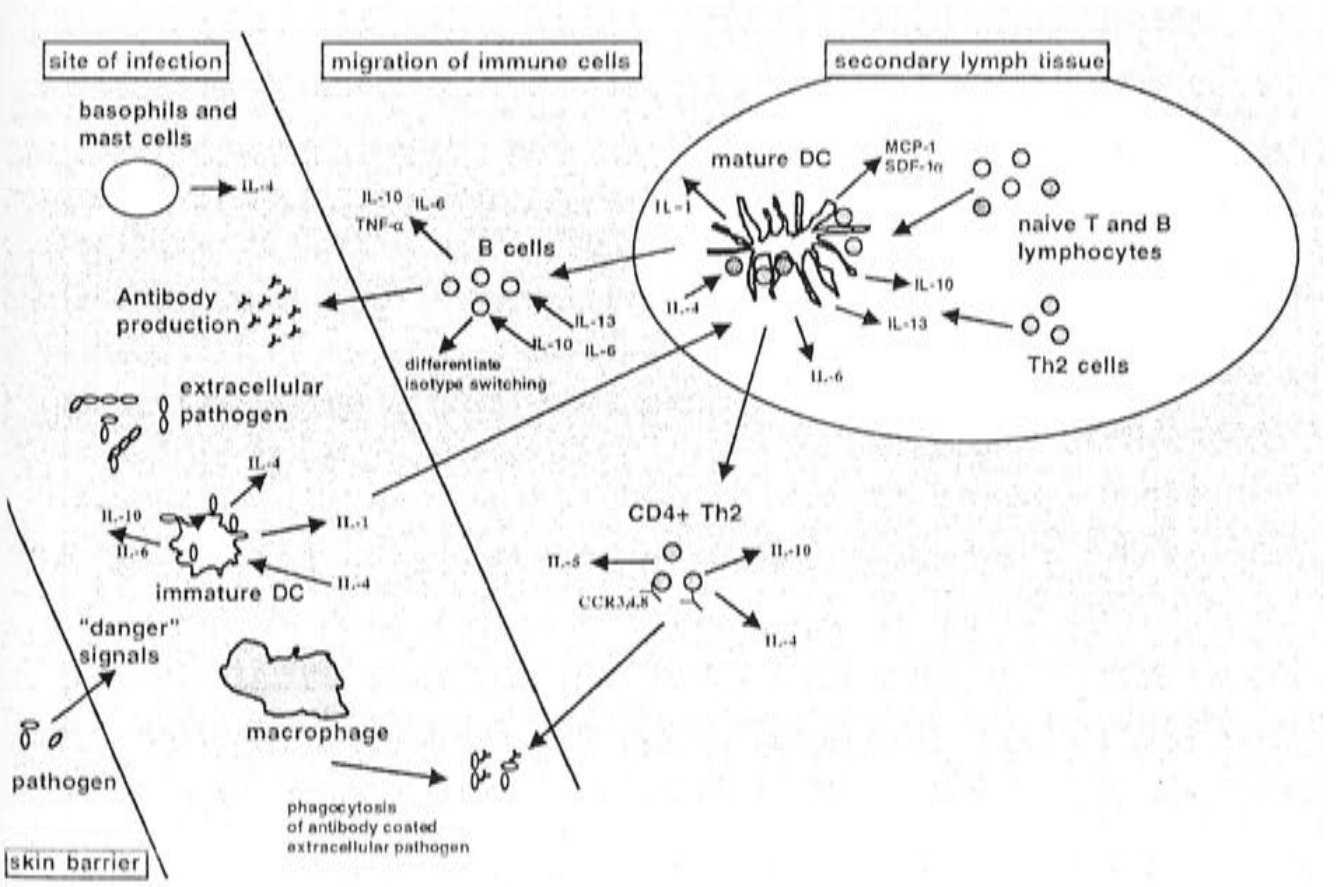

Fig. 3. **The influence of dendritic cells on the development of humoral immunity.** Following infection by extracellular pathogens, a combination of danger signals, effector cytokines, and phagocytosis of the pathogen triggers maturation events in immature dendritic cells. This maturational process leads to enhanced antigen processing, increased surface expression of MHC molecules and co-stimulatory molecules, and secretion of chemoattractants which act to recruit other leukocytes. Dendritic cells also up regulate secretion of effector cytokines, including IL-1, IL-4, IL-6, IL-10 and IL-13. The maturing dendritic cells then migrate to lymphoid tissues where they present antigen to naïve T cells and pass non-processed antigen to B cells through exosomal transfer. The T cells proliferate following both interaction with MHC and co-stimulatory molecules on the dendritic cells and stimulation by effector cytokines, and become activated Th2 cells. The B cells are stimulated to differentiate and produce antibodies. Th2-type effector cytokines, including IL-4, IL-5, IL-6 and IL-10, are secreted by the activated Th2 cells and B cells and act, in turn, to promote this pathway of adaptive immunity by stimulating further populations of T and B cells and immature dendritic cells.

the human plasmacytoid $CD11c^{-}CD4^{hi}$ DC, which secrete large amounts of IFN-α (42).

The levels of IL-12 that are released by DC are, in turn, tightly regulated by the cytokine milieu and other activation signals (38). A combination of IFN-γ, GM-CSF and IL-4, plus other activation stimuli, appear to be the most potent inducers of IL-12 secretion from $CD8\alpha^{+}$ DC. The nature of the pathogens which interact with DC are also key determinants of IL-12 secretion levels (12). Many intracellular pathogens induce IL-12 (17, 43, 44), whereas extracellular pathogens, such as helminths (45) and *Schistosoma manosoni* eggs (46), inhibit IL-12 production. The importance of the determinant role played by DC for the ultimate expression of adaptive immune responses is perhaps best illustrated by the variety of pathogens which have evolved immune escape mechanisms. For example, intracellular parasites such as HIV (47), *Leishmania major* (48), and measles virus (49), may all fail to induce IL-12 production and effective cell mediated Th1 immunity.

Many studies have focused on mechanisms of Th1 cell induction via IL-12 produced by DC. However, significant questions remain as to the nature of Th2 cell induction by DC and the source and influence of IL-4 on the DC-T cell interaction. It is clear that basophils and mast cells present at sites of infection can secrete IL-4 (Fig. 3), however there appears to be a requirement for antibody-mediated cross-linking of their receptors, indicating that an adaptive immune response must have already been generated (13). Of particular interest is the recent finding that certain pathogens may actually trigger IL-4 secretion from DC themselves. Thus, *Candida* in the form of hyphae, but not as yeast cells, down regulate IL-12 secretion by DC, but increase their secretion of IL-4 and, to a lesser extent, IL-10 (35). Consequently, protective Th1-type immune responses are generated against yeast cells, whilst hyphae elicit non-protective Th2-type responses. This example highlights the importance of the nature of the pathogen, together with early events during the process of infection, in directing the type of immune response which is generated.

Interactions of DC, Th2 cells and B cells are particularly important in situations where DC have been directed to secrete minimal levels of IL-12. Distinct DC subtypes have been shown to differentially regulate B cell responses. Langerhans cells (epidermal) and CD14-derived interstitial (dermal) DC enhance proliferation of CD40-activated B cells and induce differentiation of memory B cells. However, only interstitial DC induce naive B cells to differentiate into IgM-secreting cells (50). Interestingly, these DC also capture and retain unprocessed antigen, which is then transferred to naïve B cells resulting in the initiation of specific antibody responses (50). It has also been suggested that murine myeloid DC regulate antibody class switching, a function also associated with Th2 cell development and activity (40, 51, 52).

Th2-type cytokines, including IL-4, IL-6, IL-10, IL-13, have been shown to play important roles in enhancing B cell and DC interactions. IL-13 appears to be an essential factor promoting interdigitating DC (IDC)-induced antibody secretion and also mediates Th2 cell development (53). This factor also enhances surface expression of CD23/FcεRII and class II MHC antigens on resting B cells and stimulates B cell proliferation (54). IL-10 acts to modulate proliferation of CD40-activated B cells, naive B cells, memory B cells and germinal centre B cells, in addition to its role in enhancing antibody production. IL-10 is also a major antibody subclass switch factor and, together with TGF-β, forms a potent combination which promotes IgA isotype switching (50, 55, 56).

It is still unclear whether discrete DC lineages have a pre-determined immunoregulatory function or whether single, naïve progenitor DC circulate at a basal level in healthy individuals, taking up residence in the periphery or lymphoid tissues and reacting to stimuli from the surrounding microenvironment. These would act then either to retain the DC in lymphoid tissues or enable them to survey surrounding peripheral sites. It is clear, however, that continuing stimulation of adaptive immune cells by DC results in production of effector cytokines by activated T and B cells and that these factors promote the expression of appropriate immune responses against invading pathogens. A challenge which remains is to determine which combinations of antigen- and cytokine-stimulated progenitor cells give rise to the assortment of DC subtypes which act ultimately to drive different adaptive immune responses. The resultant information will be valuable not only for improved understanding of immune regulation by DC, but also for the development of more effective vaccines against a variety of pathogens.

Cytokine regulation of cell migration in the innate and adaptive immune response

Cytokines play a key role in controlling immune cell migration, acting initially to retain DC at the site of infection for 2–3 hours, then to promote their migration to lymphoid organs where they attract T cells. Finally, cytokines stimulate migration of Th1 and Th2 cells to sites of infection. Cytokines achieve this by stimulating the expression of a range of adhesion molecule selectins and integrins, chemokines, and chemokine receptors on the DC and adaptive T lymphocytes.

It has been shown, among proliferating murine DC, that chemokine receptor expression of CCR1 is upregulated immediately (ie. by 30–60 min) after inflammatory stimulation by LPS or TNF-α (Figs. 2 and 3). In this way, DC are attracted to sites of infection through

a gradient of inflammatory chemokines including macrophage inflammatory protein (MIP 1-α) (57). The expression of CCR1 is subsequently down regulated by 2 hours, but then progressively increased, peaking at 4 to 6 hours after stimulation (58). The short period during which CCR1 is down regulated enables these cells to remain at the site of infection. Here, the DC sample antigen and amplify the inflammatory response by secreting chemokines such as MIP-1α and MIP-2, which attract leukocytes to the site of infection (57). The antigen- and cytokine-stimulated DC then migrate to lymphoid organs once the levels of CCR1 expression rise again.

A number of studies have suggested roles for effector cytokine stimulation of DC in promoting secretion of various T cell-attracting chemokines. These factors differentially attract activated, memory, or naive CD4+ and CD8+ T cell subsets, and mediate their effects in the lymphoid tissues where their expression by DC peaks at the time of maturation (approximately 24 hrs after stimulation). CC-chemokines (MIP1-α, MIP1-β and RANTES) and CXC-chemokines (Crg-2, or IP-10, and mig) preferentially attract Th1 cells (Fig. 2) (59). MCP-1 and SDF-1α attract both Th1 and Th2 cells (59), although SDF-1 preferentially attracts the latter together with naïve T cells (Fig. 3) (60). Interestingly, the CC-chemokine, DC-CK1, which is activated by IL-4 but not IFN-γ stimulation of DC, attracts naïve T cells (61). RANTES and MCP-1 have been found to act predominantly on activated/memory stages of T cells (62, 63). Importantly, MCP-1 directly stimulates IL-4 production by T cells (64, 65), thereby enhancing Th2 cell development (66, 67). Over expression of MCP-1 is also associated with defects in cell-mediated immunity (68), whilst MCP-1-deficient mice are unable to mount Th2 responses (69). Together, these results suggest a determinant role for Th1 and Th2-type cytokines in the development of adaptive immune responses. Thus, IFN-γ appears to stimulate DC to secrete chemokines which preferentially attract activated/memory Th1 cells, whereas IL-4 stimulates these cells to produce factors preferentially attracting naïve T cells.

Chemoattractants also have a regulatory role in effector cytokine expression, acting here to support chemotaxis of particular adaptive Th subsets. For example, MCP-1,-2,-3,-4 and C5a, but not SDF-1, MIP-1α, RANTES or eotaxin, may each inhibit IL-12 production by DC in response to stimulation with IFN-γ, *Staphylococcus aureus* and Cowan strain 1 (SAC) (70).

IL-12, transforming growth factor beta (TGF-β), IFN-γ, and IL-4 also play important roles in directing specific Th cell subsets to sites of infection by modulating expression of specific chemokine receptors, such as CXCR3 and CCR5 on Th1 cells (Fig. 2) and CCR3, CCR4 and CCR8 on Th2 cells (Fig. 3) (71–74). Possession of these receptors enables the cells to respond to different chemotactic signals. Thus, IP-10, CXCR3 ligand and cutaneous lymphocyte-associated antigen (CLA) each act to recruit Th1 cells (75, 76). Conversely, MDC and I-309 (CCR8 ligand) preferentially attract Th2 cells (77). Interestingly, IL-12 promotes Th1-type immune responses by inhibiting the secretion of the Th2 cell-attracting chemokines, I-309 and MDC, by Th1 cells (78).

Cross-regulation of adaptive and innate immunity by cytokines

In addition to their determinant roles in the induction of immune responses, cytokines and chemokines, in this case produced largely by T cells, clearly play a pivotal role in immune regulation. A wide range of mechanisms are involved, including alteration of the expression of MHC molecules (79, 80), adhesion molecules (81) and costimulatory molecules (82), and direct activation or deactivation of immune cells (83, 84). These changes may lead to the activation of cellular immune responses involving NK cells and CTL, and antibody-mediated clearance of pathogens (Fig. 4) (85).

The realisation that different subsets of CD4+ T cells were distinguishable by their ability to produce discrete patterns of cytokines (86) provided a rational explanation for the early observations of Parish and colleagues showing that qualitatively different immune responses may be elicited in response to antigen challenge (87). As discussed above, many different factors influence the nature of an immune response, however cytokines secreted by T helper cells are critical regulatory molecules. As has been well documented, the Th1 cell subset secrete cytokines usually associated with inflammation, such as IFN-γ and TNF-α, and induce cell-mediated immune responses. The Th2 subset produce factors such as IL-4, IL-5 and IL-13 that provide signals for the differentiation and proliferation of B cells and thus stimulate humoral immune responses. This Th1/Th2 paradigm may now be regarded as too simplistic and has thrown up many inconsistencies and contradictions. Indeed, polarised Th1 and Th2 responses may, in reality, represent the extremes of chronic inflammation or chronic disease states (14). Helper T cells and clones which appear to secrete mixtures of both Th1 and Th2 cytokines have recently been identified and termed "Th0" cells (88, 89). These may be precursors for Th1 and Th2 cells or, alternatively, represent a separate lineage, possibly acting in the elimination of pathogens against which concomitant and appropriately regulated humoral and cell-mediated immune responses are required. Despite these developments, decisive advances in our understanding of immune regulation resulted from the development of the Th1/Th2 paradigm and many of its tenets remain valid.

One such issue is the important roles played by Th1- and Th2-

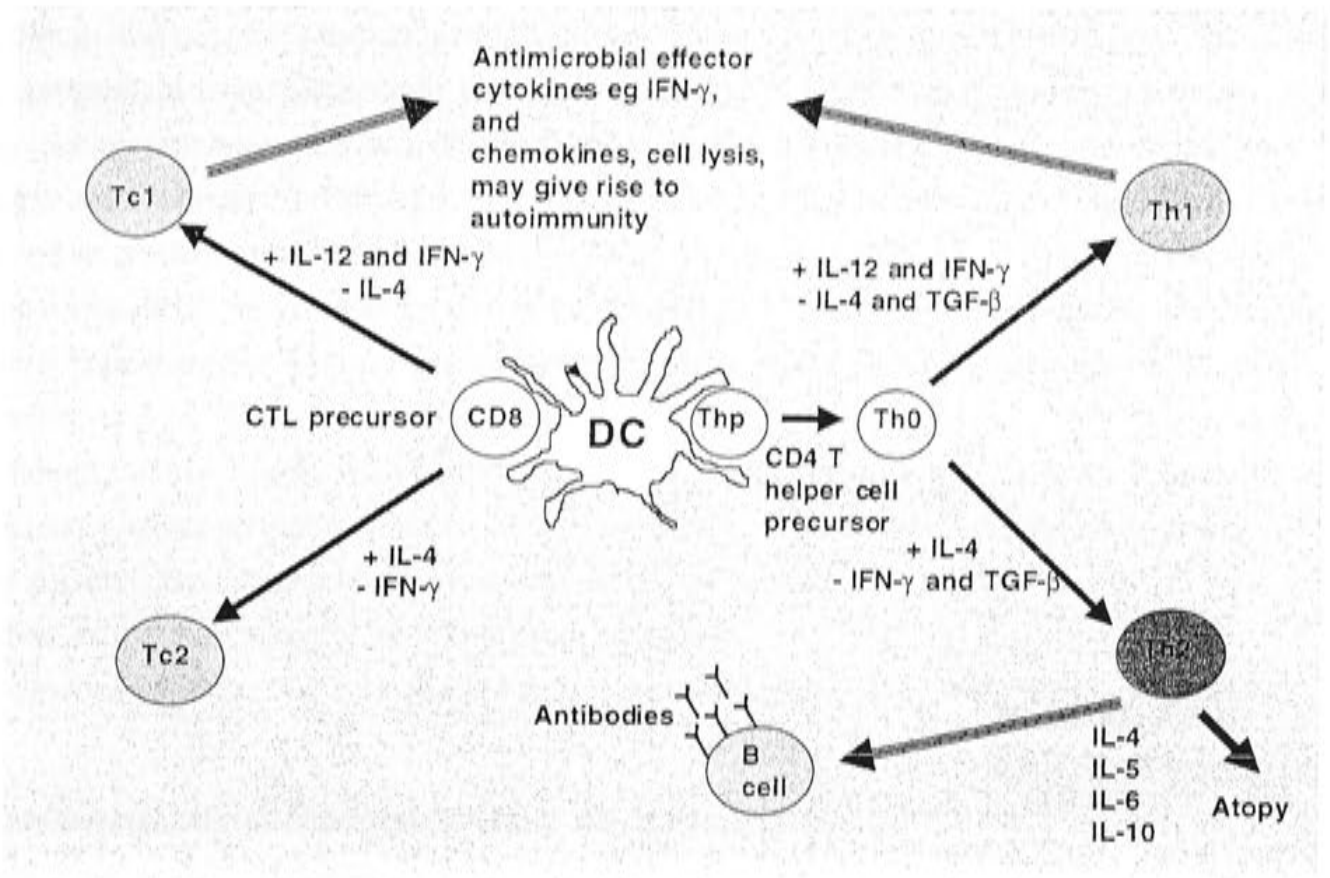

Fig. 4. **Cytokine regulation of anti-microbial immune responses and immuno-pathologies.** The types of cytokines which are produced during the early stages of an immune response are major determinants of the types of immune cells that are generated as a result. Cytokines may be divided into two categories: those driving T cell development towards either a Th1/Tc1 phenotype (associated with cell-mediated immunity), or those which promote a Th2/Tc2 phenotype (leading to the production of antibodies by B lymphocytes). At the earliest stages of the immune response, these factors are produced by a variety of cells, including macrophages (IL-12), NK cells (IFN-γ) and mast cells/basophils (IL-4). As the response develops, T cells become the major producers of these factors. In addition, Th1-associated cytokines reciprocally down regulate the development and activity of Th2-associated responses and vice versa. Most virus infections stimulate strong antiviral Th1/Tc1 responses, which act to resolve the infection. TGF-β, produced by a putative "Th3" subset, may down regulate the development of both Th1 and Th2 subsets and inhibit inflammatory pathologies (e.g. autoimmunity and allergies). Solid arrows indicate cell lineage development, streaked arrows indicate regulatory/effector activity.

type cytokines and, indeed, other subsets, in reciprocally cross-regulating each other's development and maintaining their phenotype and function (Fig. 4). Indeed, whilst these cells appear to be major determinants of cell-mediated and humoral immune responses against invading pathogens as outlined above, their dysregulation can have disastrous consequences. Thus, Th1 cells may cause autoimmune disease if improperly regulated (90, 91), whilst allergic conditions result from the over-production of factors such as IL-4 and IL-5 by Th2 cells (92, 93). Thus, IFN-γ secreted by Th1 cells may inhibit the development of Th2 cells (94) and immune responses mediated by antibodies of the IgG1, IgE and IgA subclasses. Conversely, IL-4 and IL-10 produced by Th2 cells may block the development and activation of Th1 cells and macrophage activity (13, 88). It has been proposed that a major function of Th2 cells may be to down regulate Th1 cell-mediated immune responses and limit their damaging effects (29).

Further regulatory subsets, distinct from Th2 cells, have also been proposed or identified in order to explain novel experimental phenomena. Some of these, often termed Th3 cells (95), are able to inhibit inflammatory diseases mediated by Th1 cells (95–97) and Th2 cells (95, 97), and this is thought to be related to their expression of TGF-β. This immunosuppressive or anti-inflammatory factor was previously shown to inhibit the development of both Th1 and Th2 cell subsets (98, 99).

Cytokine-mediated feedback regulation of innate immune cells

Cytokines expressed during an adaptive immune response may, in addition to regulating the expression of Th cell phenotypes, also act on innate cells as a feedback mechanism for the purposes of maintenance or amplification of immunity. Thus, IFN-γ may act in synergy with CD40 activation of DC, increasing IL-12 production and promoting cell-mediated immunity and further IFN-γ secretion. IFN-γ, in turn, activates macrophages to express bacteriocidal activity and phagocytosis, to undergo an oxidative burst and to produce nitric oxide and secrete IL-12 and TNF-α, both of which support further Th1 cell development (100).

The role of IL-4 in directly priming for differentiation of naive CD4+ T cells into Th2 is well documented. Both IL-4 and IL-13 may also influence Th2 development by acting directly on DC to downregulate IL-12 secretion (100). We have recently reported that

IL-4 may also support development of Th2 responses by acting on DC progenitors to maintain high levels of expression of FcγR and macrophage mannose receptors during maturation (101). These elements are important for binding extracellular pathogens and are known to down regulate IL-12 production (102). In contrast, IFN-γ stimulation of DC progenitors results in reduced levels of FcγR and macrophage mannose receptor expression and promotes high levels of expression of Th1 cell-attracting chemokines such as RANTES (reviewed in 101). Finally, IL-10 produced by DC, Th2 cells and B cells may also act on innate immune cells to support Th2-type adaptive immune responses through its activities both as an endogenous and an exogenous regulator of IFN-α production (103).

Virus-encoded homologues of cytokines and their receptors

The critical importance of cytokines and chemokines in innate and adaptive immune defenses is illustrated by the expression of a variety of inhibitors and homologues by many different pathogens, particularly viruses (104). Several poxviruses, including cowpox, variola (smallpox), ectromelia (a rodent pox virus), and myxoma (rabbit pox) viruses, encode for proteins which have cytokine or cytokine receptor homology. These molecules have apparently evolved to aid the virus in evading host immunity (105). An outstanding example of such a strategy is provided by cowpox virus, which encodes three TNF receptor homologues, termed, CrmB, CrmC, and CrmD which may all bind TNF-α, but with different affinities. Unlike CrmC, the TNFR homologues CrmB and CrmD also bind TNF-β. CrmB is expressed early during infection whereas CrmC and CrmD are expressed at later times during infection (106). The exact function of these TNFR homologues is unclear although it has been demonstrated that the deletion of the CrmB protein (also termed T2) from myxoma virus markedly decreases virus pathgenicity in rabbits (107). This protein also inhibits virus-induced apoptosis in rabbit T lymphocytes (108). Herpesviruses are another group which have evolved an extensive array of strategies for immune evasion, some of which have developed to counteract antiviral cytokine activity. For example, Epstein-Barr virus (EBV) encodes a 17kDa product, termed BCRF1, which has 78% homology with human IL-10 and which is expressed during the lytic phase of EBV infection. Both recombinant IL-10 and BCRF1 inhibit macrophage and monocyte activation, as well as IFN-γ expression by NK cells and T cells, resulting in inhibition of the up regulation of MHC molcules by antigen-presenting cells, nitric oxide production and other antiviral pathways (109). Another herpesvirus, human cytomegalovirus (HCMV) inhibits both IFN-α and IFN-γ-induced expression of class I and II MHC molecules by decreasing JAK1 and p48 expression, thus down regulating the JAK/STAT signalling pathway (110).

In addition, several viruses have developed strategies to modulate chemokine activity (105). These include (i) the production of molecules which mimic chemokines themselves, (ii) the expression of chemokine receptor homologues, which presumably compete for chemokine binding, and (iii) the production of chemokine-binding proteins to inhibit host chemokine activity.

In vivo studies of the immunoregulatory and antimicrobial activity of cytokines

Numerous approaches have been used to study the role of cytokines both in regulating immune responses and in protecting the host against infection. The use of transgenic and gene knock out mice has proved extremely useful in this regard. One approach we have used has been to engineer recombinant viruses to express genes encoding cytokines and chemokines and to use these constructs to study the effects of these factors on host immune responses. Some of our key results are summarised in Table 1.

During replication *in vivo*, the recombinant viruses produce the encoded factor which is secreted from infected cells. The location and extent of virus replication thus determines the levels and sites of cytokine or chemokine production. This approach has enabled us to study the effects of these molecules on the development of both innate and adaptive immune responses and also their role in mediating immunity to infection. Many of the encoded factors, including the cytokines IL-2 and IL-12, and the chemokines Mig and Crg-2, showed potent antiviral activity *in vivo*. Each of these factors induced particular antiviral host defense mechanisms, whilst for other encoded factors (e.g. IFN-γ, TNF-α and CD40 ligand), a direct antiviral effect was demonstrated (111). The use of mice rendered genetically deficient for IFN-γ or IFN-γ receptor expression allowed us to show that both IL-2 and IL-12 mediated their antiviral activity largely through the induction of host IFN-γ production (112, 113). The antiviral activity of IL-2 lay in the ability of this factor to activate and attract NK cells to sites of infection and to stimulate their production of IFN-γ, which, in turn, limited the growth of the recombinant virus. IL-12, which also showed potent antiviral activity when expressed by rVV, is a strong stimulator of IFN-γ production. As early as 1 day after infection, VV encoding IL-12 was recovered at titers approximately 100-fold lower than a control rVV in normal mice. However, IL-12 itself may also exert antiviral activity, as dem-

Known functions of cytokines and their effects *in vivo* when expressed by recombinant vaccinia viruses

Cytokine	Major known function	Effect on immunity when expressed by rVV	References
Type 1 factors			
IL-2	T cell growth factor, activates B cells, monocytes and NK cells	Attenuates growth of virus, stimulates natural killer cells to produce IFN-γ	(121–123)
TNF-α	Antiviral activity *in vitro*, activates cytotoxic T cells	Limits growth of vVV, allowing T cell-deficient mice to resolve infection.	(124)
IFN-γ	Up-regulates MHC molecules on antigen presenting cells, anti- viral activity	As above.	(125)
IL-12	Helps initiate type 1 responses, promotes IFN-γ production	Limits growth of rVV. stimulates IFN-γ secretion, down regulates type 2 responses	(111, 113)
Type 2 factors			
IL-4	Regulates antibody responses, especially IgE. Down-regulates type 1 responses	Increases pathogenicity of rVV, down-regulates CTL responses	(119)
IL-5	Regulates antibody production	Stimulates antigen specific mucosal IgA responses	(126)
IL-6	Involved in the maturation of B cells to ASCs	Stimulates antigen-specific systemic IgG and mucosal IgA responses	(127)
Other factors			
IL-1α	Activates T cells	Enhances memory antibody responses	(128)
IL-7	Early activator of T and B cells	Stimulates T cell and non-specific antiviral immunity	(129)
CD40L	Triggers B cells to make antibody	Antiviral activity	(116)
Mig Crg-2	Recruits leukocytes to sites of inflammation	Antiviral activity leading to resolution of infection	(130)

Table 1

onstrated by the observation that mice deficient in this factor failed to mount significant antiviral CTL responses or to clear the virus efficiently, despite unimpaired IFN-γ expression (114). Thus, in addition to its regulatory activity in Th1 cell development, IL-12 may also play an important role as an antiviral mediator, both through the activity of IFN-γ and independently of this factor.

TNF-α, which is produced predominantly by macrophages, also displays potent antiviral activity when expressed by recombinant VV. Studies using mice deficient in TNF receptor 1 (P55) and/or TNF receptor 2 (P75) showed that both receptors were involved in antiviral activity (115). Indeed, as outlined above, several pox viruses have naturally acquired genes encoding for proteins which have homology with these TNF receptors and which are thought to be important in viral pathogenesis (107).

Expression of the T cell surface molecule CD40 ligand in rVV also produced, somewhat unexpectedly, a highly attenuated virus phenotype *in vivo* (116). The attenuation of VV-CD40 ligand in wild type mice was, however, completely reversed in CD40-deficient mice. Interestingly, CD40 expression is not restricted to B cells, macrophages and DC, but is also found on tumor cells and may be induced on fibroblasts incubated with IFN-γ and TNF-α. Thus, we have speculated that CD40 ligand/T cell-mediated activation of CD40 on virus-infected cells may act to limit the production of progeny virus, possibly through apoptosis of infected cells.

In addition to its roles in promoting Th2 cell development and down regulating Th1 cell differentiation, IL-4 may influence the differentiation of other lymphocyte populations. For example, *in vitro* stimulation of naïve CD8+ T cells in the presence of IL-2, IL-12 or IFN-γ generates classical cytotoxic T cells which express IFN-γ (also termed Tc1 cells), whereas CD8+ cells stimulated in the presence of IL-4 develop a Tc2 phenotype resulting in their expression of IL-4, IL-5, IL-6 and IL-10, often with markedly reduced cytotoxic activity (117, 118). In our studies, expression of IL-4 by recombinant poxviruses markedly increases their pathogenicity via down regulation of the antiviral CTL response and IFN-γ production (119). Limiting dilution analysis revealed a 12-fold reduction in CTL precursor frequency in VV-IL-4 infected mice. Others have shown that treatment of activated Tc1 cells with IL-4 also results in defective IFN-γ, TNF-α and IL-2 expression. Although IL-4 treated Tc1 cells retained short-term *in vitro* cytotoxic activity, they failed to proliferate in response to antigen stimulation, compromising their long-term functional capability to control infection (118). It has recently been shown that NK cells cultured in the presence of either IL-12 or IL-4 may differentiate into "NK1" or "NK2" cells respectively, with distinct patterns of cytokine secretion similar to Th1 and Th2 cells, although this does not appear to effect their *in vitro* cytotoxic activity (120). Thus, expression of IL-4 early in infection, can dramatically down regulate, or even prevent, induction of protective immune responses to infectious pathogens.

These examples illustrate that the profiles of cytokines which are induced early in infection are of critical importance for the immunological control of infection. Cytokines and chemokines not only regulate the types of cells which are activated, but many of these factors also have direct antiviral effector function or the capacity to stimulate immune cells to secrete further antiviral molecules. Cytokines may also act as reciprocal messengers to co-ordinate innate and adaptive immune responses against infectious agents. The initial encounter with a pathogen elicits an innate response that will, to a large extent, determine the nature of the subsequent adaptive response. The nature of the invading pathogen, the route of infection and the genetic background of the host all play a role in influencing the types of responses which are induced. Conversely, cytokines produced by cells of the adaptive immune system may also, via feedback mechanisms, influence the types of effector activity expressed by innate immune cells. This highly developed regulation of innate and adaptive immune responses has evolved to ensure that the most efficient immunological effector mechanisms are unleashed on any particular infectious agent among the wide variety of pathogens which are likely to be encountered.

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APPENDIX 1B

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IFN-γ Regulates Murine Interferon-Inducible T Cell Alpha Chemokine (I-TAC) Expression in Dendritic Cell Lines and during Experimental Autoimmune Encephalomyelitis (EAE)

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Hamilton NHR, Banyer JL, Hapel AJ, Mahalingam S, Ramsay AJ, Ramshaw IA, Thomson SA. IFN-γ Regulates Murine Interferon-Inducible T Cell Alpha Chemokine (I-TAC) Expression in Dendritic Cell Lines and during Experimental Autoimmune Encephalomyelitis (EAE). Scand J Immunol 2002;55:171–177

Murine interferon-inducible T cell alpha chemokine (I-TAC) is a potent non-ELR Cys-X-Cys (CXC) chemokine that predominantly attracts activated T lymphocytes and binds to the receptor CXCR3. Using semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) we analysed murine I-TAC expression in two different progenitor dendritic cell (DC) lines, MTHC-D2 and JAWS II which were exposed to various cytokines, and Con A-activated splenocytes from a panel of knockout mice. Analysis of the progenitor DC lines and Con A cultures demonstrated that murine I-TAC is primarily regulated by interferon (IFN)-γ via interferon regulatory factor (IRF)-1. It has been proposed that I-TAC may have a role in autoimmune diseases such as multiple sclerosis (MS). Because I-TAC appears to be secreted from antigen-presenting cells (APCs) and attracts activated T cells, we examined the level of murine I-TAC mRNA in the central nervous system (CNS) of wild-type and IFN-γ-receptor knockout (IFN-γR$^{-/-}$) mice with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide-induced experimental autoimmune encephalomyelitis (EAE). Peak I-TAC expression was detected in wild-type mice on day 14 when the mice begin to recover, whereas very low levels of I-TAC were detected in the CNS of IFN-γR$^{-/-}$ mice which develop severe EAE and die. The expression characteristics of murine I-TAC suggest an important mediator of immune cell communication that could augment vaccines and autoimmune therapies.

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INTRODUCTION

Chemokines are small proteins that mediate the recruitment and activation of leucocytes and other cells during immune responses. How various chemokines influence dendritic cell (DC)/T-cell migration during early immune events has recently received increased attention, as the importance of chemokines during these events has become apparent. A number of key chemokines have already been shown to be involved in regulating the migration and maturation of DCs as they travel to the draining lymph nodes and as they influence early T-cell events in the lymph [1].

Relatively recently the human non-ELR CXC chemokine ('E-L-R' refers to the amino acid motif required for recruiting neutrophils), interferon-inducible T cell alpha chemokine (I-TAC), also known as H174 [2], β-R1 [3], SCYB11 [4], IP-9 and CXCL11 [5], was identified and found to be a potent chemoattractant for activated T lymphocytes,

NK cells [6, 7] and monocyte-like cells [8]. Human I-TAC is homologous to IP-10 and monokine induced by IFN-γ (MIG), and like these chemokines, binds to the chemokine receptor CXCR3 but with a significantly higher affinity suggesting a key role in T cell-mediated immunity [6, 9]. Expression of human I-TAC has been detected in cultured primary monocytes, cultured foetal astrocytes, microglial cell line [6], astrocytoma cells [3], atheroma-associated cells [10], bronchial epithelial cells [11], polymorphonuclear neutrophils [7] and keratinocytes [12]. Moderate expression has also been detected in human central nervous system (CNS) [8], pancreas, lung, thymus and spleen tissues [6]. *In vitro* studies have demonstrated that the expression of human I-TAC appears to be upregulated following the exposure to mainly interferon (IFN)-α, IFN-β or IFN-γ plus other cell regulators such as interleukin (IL)-1, lipopolysaccharide (LPS) or tumour necrosis factor (TNF)-α [3, 6].

The increased I-TAC expression in human astrocytes and its ability to attract activated T cells has led some researchers to propose that I-TAC may have a role during the induction and pathophysiology of neuroinflammatory disorders such as multiple sclerosis (MS) [6, 8]. An animal model for MS, experimental autoimmune encephalomyelitis (EAE), is a CNS-demyelinating disease induced in susceptible mice, rats, guinea pigs, or nonhuman primates following immunization with myelin-derived antigens, peptides, or following the transfer of autoreactive $CD4^+$ T-cell lines or clones [13]. During the initiation of EAE, activated-Th1 autoreactive T cells appear to infiltrate the CNS by an antigen- and apparently chemokine-independent process which leads to the increased expression of a range of proinflammatory cytokines within the CNS [14, 15]. These proinflammatory cytokines, among other effects, upregulate the expression of a suite of chemokines which have been implicated in various stages of EAE disease [15–21]. Confirmation of a key role for I-TAC during EAE has been slow because of the delay in the identification of a rodent I-TAC homologue. Murine I-TAC, however, has been recently identified, but its role during EAE has not been examined [22, 23].

In this study, we report that murine I-TAC is expressed from two progenitor DC lines, JAWS II and MTHC-D2. Murine I-TAC expression in Con A-activated splenocytes from a panel of knockout mice allowed us to further delineate the cytokine regulation of murine I-TAC expression. Murine I-TAC was primarily regulated by IFN-γ but not IL-4 or TNF-α. Finally, given the central role of antigen-presenting cells (APCs) in the CNS of animals with EAE and the fact that I-TAC is expressed from DCs and is IFN-γ regulated, we analysed murine I-TAC expression in the CNS of both wild-type and IFN-γR knockout mice during EAE. Taken together, these results suggest that I-TAC may have an important regulatory role during the T cell-mediated immunity.

MATERIALS AND METHODS

Mice. Specific pathogen-free 6–8-week-old female mice were obtained from the Animal Breeding Facility, John Curtin School of Medical Research, Canberra, Australia. IFN-α/β$R^{-/-}$, IFN-γ$R^{-/-}$ and IFN-α/β-γ$R^{-/-}$ double knockout mice homozygous for the disrupted gene(s) and backcrossed onto the 129/Sv background, were obtained from Dr Michel Aguet, University of Zurich [24–26]. Interferon regulatory factor (IRF)-$1^{-/-}$ mice backcrossed onto the 129/Sv background were obtained from Prof Charles Weissman, University of Zurich [27]. $CD40^{-/-}$ mice were obtained from Prof Hitoshi Kikutani, Osaka University [28]. TNF-IR-II$R^{-/-}$ double knockout mice were obtained from Dr Jacques Peschon, Immunex Research and Development Corporation [29]. Two sources of age-matched null homozygote (C57/BL6 × 129/Sv) mice were used as controls for these assays (Max-Planck Institute for Immunbiologie, Freiburg, Germany and University of Zurich, Switzerland) as described elsewhere [30, 31].

DC cell lines and Con A blasts. The progenitor DC cell line, JAWS II (ATCC, CRL-11904), isolated from $p53^{-/-}$ mice, was maintained in alpha minimum essential medium with ribonucleosides and deoxyribonucleosides containing L-glutamine (Gibco/BRL Paisley, UK) and supplemented with 20% foetal bovine serum (FBS) (Gibco/BRL), 1 mM sodium pyruvate and 5 ng/ml murine GM-CSF (Sigma, St. Louis, MO, USA).

The DC progenitor cell line, MTHC-D2, whose features are summarized by Banyer and Hapel [32], was subcloned from MTHC lines described previously [33]. This cell line was maintained in Iscove's modifed eagle medium (IMEM; Gibco/BRL) supplemented with 10% FBS (Gibco/BRL), 2 mM glutamine (Sigma), and 50 U/ml GM-CSF.

JAWS II and MTHC-D2 cell lines were stimulated for 24 or 48 h with TNF-α (500 U/ml) and GM-CSF (5 μ/ml), and either IFN-γ (2 U/ml) or IL-4 (50 U/ml). Aliquots of these cell samples were then activated for a further 24 h by CD40 cross-linking using CD40 Ab (25 μg/ml) protein A column-purified from supernatant of cultured FGK45.5 cells [34].

Con A blasts from mice were generated from RBC-lysed splenocytes ($2–5 \times 10^6$ cells/ml) by culturing for 2 days in F15 MEM containing 10% FCS (Gibco/BRL) and Con A (2 μg/ml, Pharmacia Biotech, St. Quentin, Yvelines, France).

Reverse transcription–polymerase chain reaction (RT–PCR). Murine I-TAC expression was analysed by hot-start semiquantitative RT–PCR. Briefly, mRNA was isolated from either cell lines (5×10^6 cells) or Con A blasts (1×10^6 cells) using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) and first strand cDNA prepared using a Ready-To-Go T-Primed First Strand Kit (Pharmacia Biotech). First strand cDNA was also produced from mRNA isolated from CNS tissue excised using standard techniques from mice with EAE. The first strand cDNAs (1 μl) from DC cell lines, Con A blasts and CNS tissue were PCR-amplified in various experiments (reaction volume 20 μl) using Taq polymerase (1.5 U/reaction, Gibco/BRL), the supplied 10× buffer, $MgCl_2$ (2 mM), dNTPs (0.2 mM) (Pharmacia Biotech), and oligonucleotide primers (2 μM). The thermal profile used was as follows: denaturing at 94 °C for 10 s, annealing at 66 °C for 20 s, and extension at 72 °C for 30 s for 30–32 cycles. Oligonucleotide primers used to amplify a 252-bp I-TAC fragment were mITAC sense, 5′-GAACAGGAAGGTCACAGCCATAGC-3′ and mITAC antisense, 5′-ATGAGGCGAGCCTGCTTGGATCTG-3′. RT–PCR products

were viewed by gel electrophoresis (3% Nuseive GTG agarose, BioWhittaker, Rockland, ME, USA). All results were standardized against murine GAPDH by amplifying a 551-bp fragment using the primers mGAPDH sense, 5′-ACCACAGTCCATGCCATCAC-3′ and mGAPDH antisense, 5′-TCCACCACCCTGTTGCTGTA-3′. The thermal profile used to amplify the GAPDH fragment was as follows: denaturing at 94 °C for 10 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s for 28 cycles. Prior to the actual assays, the thermal conditions and number of cycles used were optimized for both primer pairs so that the PCR products obtained did not proceed past the exponential phase of the PCR reaction nor exceed analysis limits. All semiquantitative RT–PCR experiments were carried out more than once.

EAE model. The peptide corresponding to amino acids 35–55 of rat MOG (^{35}Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys^{55}) was synthesized by standard f-moc chemistry and the purity determined by reverse phase HPLC (Biomolecular Resource Facility, JCSMR, ANU). Rat MOG_{35-55} (2 mg/ml in saline) was emulsified in an equal volume of CFA containing 0.5 mg/ml *Mycobacterium butyricum* and 4 mg/ml *Mycobacterium tuberculosis* (H37Ra). Each mouse received 100 µl emulsion distributed in the footpads of both hind feet and in the nape of the neck. The total dose of MOG_{35-55} was 100 µg/mouse. Immediately before and 2 days after injection of the emulsion, the mice received an i.v. injection of 300 ng of pertussis toxin (List Biological Laboratory, Campbell, CA, USA) in 250 µl of phosphate-buffered saline (PBS). The mice were monitored daily and assigned EAE severity scores as described previously [13].

RESULTS

Cytokine-induced expression of murine I-TAC in DC lines

To investigate which cytokines regulate murine I-TAC expression, we carried out semiquantitative RT–PCR analysis (Fig. 1) on mRNA isolated from the JAWS II (Fig. 1A) and MTHC-D2 (Fig. 1B) cell lines exposed to TNF-α plus IFN-γ or TNF-α plus IL-4 cytokines. These two cell lines are progenitor cells of DC isolated from different tissues and which have been transformed by two different approaches. Semiquantitative RT–PCR analysis of both the JAWS II (Fig. 1A) and the MTHC-D2 (Fig. 1B) cell lines showed that the expression of murine I-TAC was upregulated by IFN-γ/TNF-α (lanes 3) compared with the IL-4/TNF-α (lanes 2) or untreated cells (lanes 1) both of which had no detectable murine I-TAC band. These data also indicate indirectly that IL-4 and TNF-α alone do not upregulate I-TAC expression and that the key cytokine for expression appears to be IFN-γ. Northern blot analysis (not shown) of the MTHC-D2 cells confirmed the RT–PCR data and also confirmed the reported size of the full-length murine I-TAC mRNA at approximately 1.3 kb [22].

CD40 Ab-induced expression of murine I-TAC in DC lines

Synergy between IFN-γ and CD40L has been shown to enhance the expression of a number of chemokines from cervical carcinoma cells [35]. In conjunction with the cytokines above, we examined the effect of anti-CD40 Ab on murine I-TAC expression (Fig. 1). Semiquantitative RT–PCR analysis, standardized against GAPDH, showed that in the absence of IFN-γ, anti-CD40 Ab upregulated the expression of murine I-TAC in MTHC-D2 cells (Fig. 1B: lanes 1 and 2 versus lanes 4 and 5, respectively), but not in the JAWS II cell line (Fig. 1A: lanes 1 and 2 versus lanes 4 and 5, respectively). An increased expression pattern was detected in cells exposed to both CD40Ab and IFN-γ with the MTHC-D2 cell line demonstrating synergy (Fig. 1B: lane 3 versus lane 6) whereas synergy in the JAWS II was not detected (Fig. 1A: lane 3 versus lane 6). The different I-TAC expression pattern between the cell lines appears to be primarily owing to differences in the CD40 activation pathway rather than in the interferon-signalling pathway.

Expression of murine I-TAC Con A blasts from knockout mice

To further analyse murine I-TAC expression and confirm the cell line results, semiquantitative RT–PCR, standardized against GAPDH, was carried out on cDNA from inactivated and Con A-activated splenocytes isolated from a panel of knockout mice. Murine I-TAC expression in inactivated splenocyte cDNA samples from the various transgenic mice and controls was very low and not significantly different

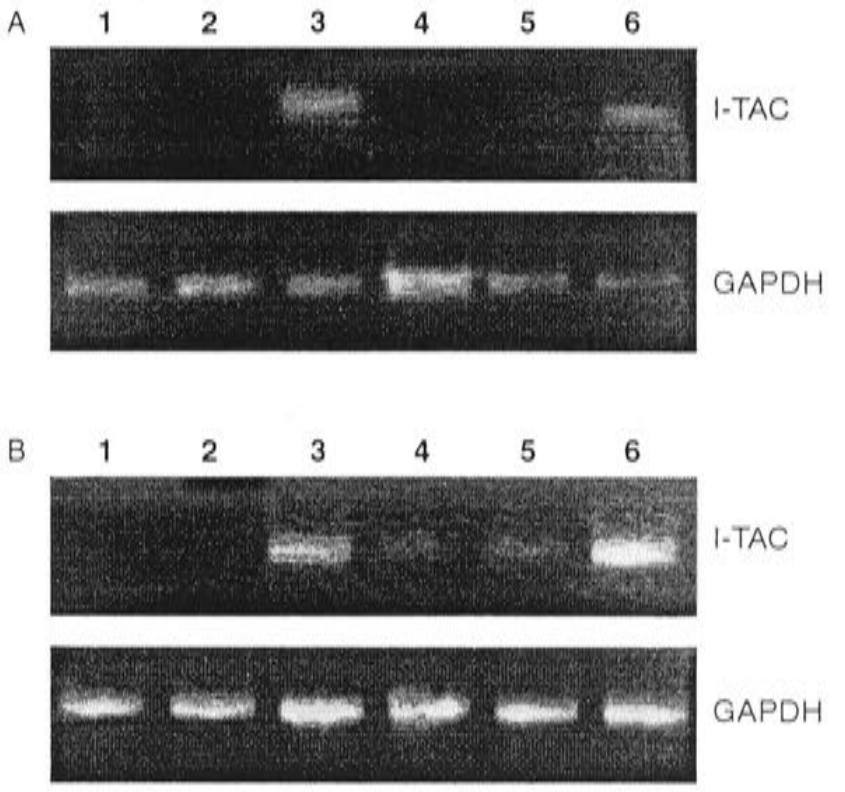

Fig. 1. Semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) murine interferon-inducible T cell alpha chemokine (I-TAC) and GAPDH mRNA expression in the JAWS II (A) and MTHC-D2 (B) cell lines without cytokine stimulation (lane 1) and following exposure to the cytokines; interleukin (IL)-4/tumour necrosis factor (TNF)-α (lane 2), interferon (IFN)-γ/TNF-α (lane 3), without cytokine stimulation plus anti-CD40 Ab (lane 4), IL-4/TNF-α plus anti-CD40 Ab (lane 5), and IFN-γ/TNF-α plus anti-CD40 Ab (lane 6).

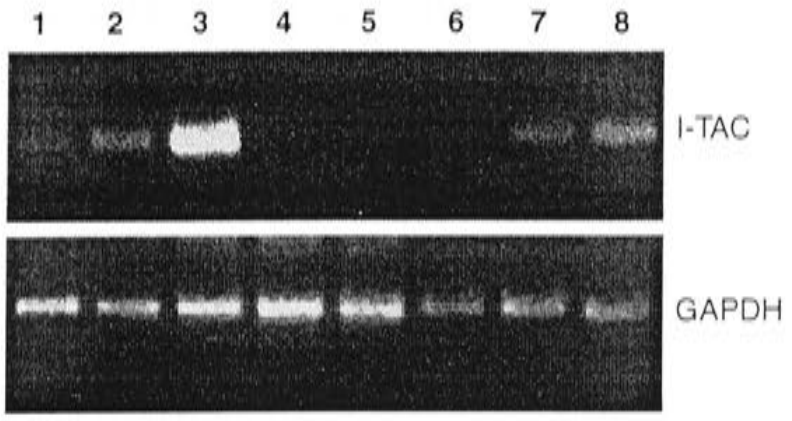

Fig. 2. Murine interferon-inducible T cell alpha chemokine (I-TAC) and GAPDH semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of Con A-activated splenocytes from a panel of knockout mice. Two sources of age-matched littermate null homozygote (129/Sv × C57BL/6) mice were used as controls (lanes 1 and 2). The panel of knockout mice were as follows: interferon (IFN)-α/βR$^{-/-}$ (lane 3), IFN-γR$^{-/-}$ (lane 4), and IFN-α/βR-γR$^{-/-}$ (lane 5), interferon regulatory factor (IRF)-1$^{-/-}$ (lane 6), CD40$^{-/-}$ (lane 7), and tumour necrosis factor (TNF)-IR-IIR$^{-/-}$ (lane 8). The cDNA samples from three individual mice in each case were obtained and then combined equally prior to PCR amplification.

(data not shown). In the Con A-activated splenocyte samples (Fig. 2), there was a significant increase in the level of murine I-TAC expression in splenocytes from IFN-α/βR$^{-/-}$ (lane 3) mice compared with control (lanes 1 and 2). These results appear to indicate that IFN-α and/or IFN-β were down-regulating murine I-TAC expression in these assays. Expression of murine I-TAC in splenocytes from IFN-γR$^{-/-}$ (lane 4), IFN-α/βR-γR$^{-/-}$ (lane 5) and IRF-1$^{-/-}$ (lane 6) mice were all significantly lower than the expression in the splenocytes from control mice (lanes 1 and 2). These results showed that the IFN-γ signalling pathway is the primary regulator of murine I-TAC mRNA expression and that IRF-1 has a significant role in murine I-TAC expression. Murine I-TAC expression in activated splenocytes from CD40$^{-/-}$ (lane 7) mice was not significantly different to the samples from control mice (lanes 1 and 2), corresponding to the *in vitro* data for the JAWS II cell line (Fig. 1A) but not to the data for the MTHC-D2 cell line (Fig. 1B). There was no significant difference between the level of murine I-TAC detected in TNF-IR-IIR$^{-/-}$ (Fig. 3, lane 8) mice compared with control mice (lanes 1 and 2), which suggests that TNF-α does not have an important role in regulating murine I-TAC mRNA, as also shown indirectly by the *in vitro* cell line data.

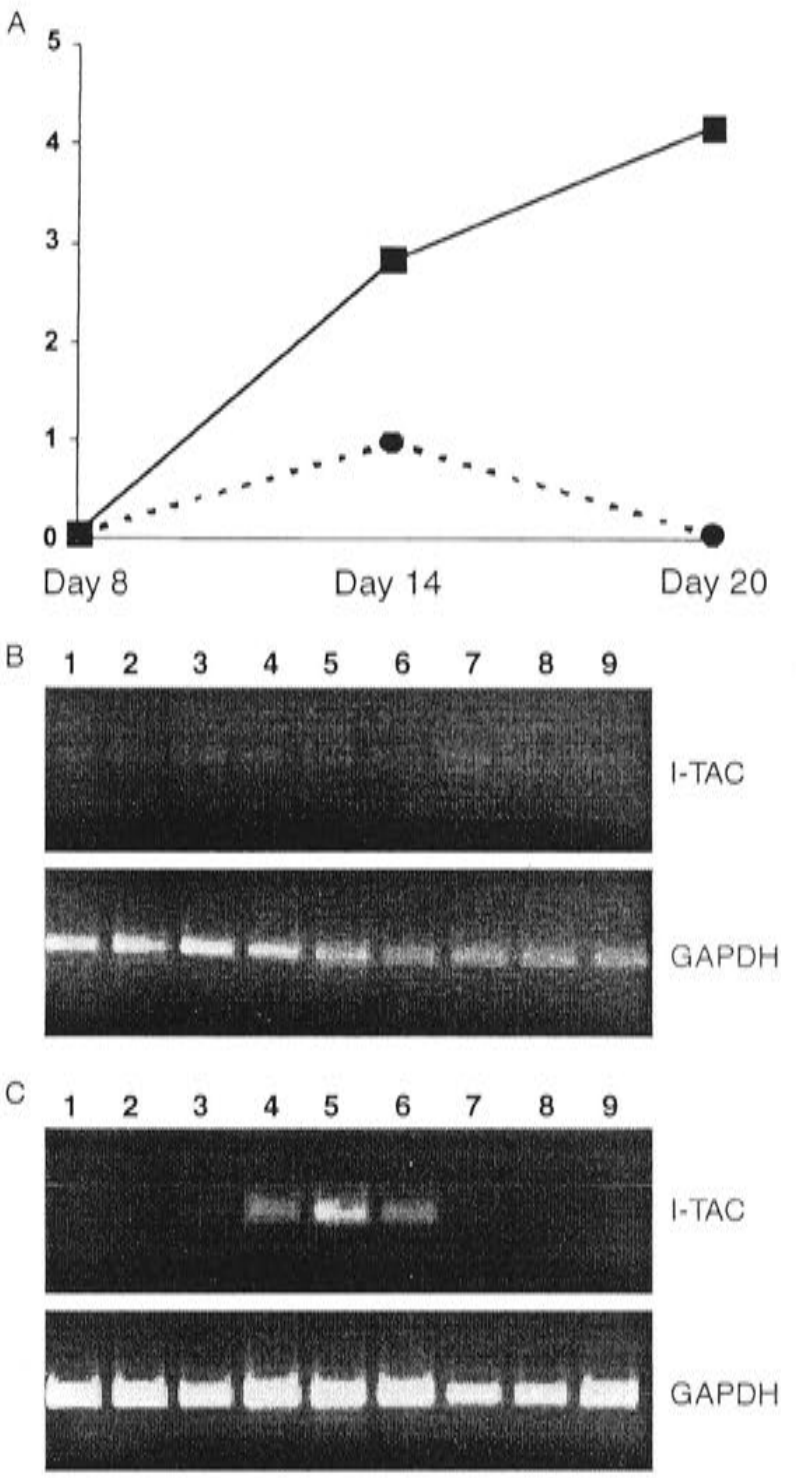

Fig. 3. Analysis of murine interferon-inducible T cell alpha chemokine (I-TAC) expression in the central nervous system (CNS) of mice with experimental autoimmune encephalomyelitis (EAE). (A) Disease scores of wild-type (circles) and interferon (IFN)-γR$^{-/-}$ (squares) mice with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide-induced EAE. (B) Semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of murine I-TAC and GAPDH mRNA CNS expression in IFN-γR$^{-/-}$ mice with EAE at day 8 (lanes 1–3), day 14 (lanes 4–6) and day 20 (lanes 7–9). (C) Semiquantitative RT–PCR analysis of murine I-TAC and GAPDH mRNA CNS expression in wild-type mice with EAE at day 8 (lanes 1–3), day 14 (lanes 4–6) and day 20 (lanes 7–9).

Expression of murine I-TAC in the CNS of mice with EAE

Previously, it has been reported that IFN-γR$^{-/-}$ mice challenged with rat MOG_{35-55} peptide develop severe EAE and die whereas wild-type control mice (IFN-γR$^{+/+}$) develop a milder disease and recover [13]. Given that I-TAC attracts activated T cells, is regulated by IFN-γ and is expressed from astrocytes in the CNS, we investigated murine I-TAC expression in the CNS of wild-type and IFN-γR$^{-/-}$ mice with MOG_{35-55}-induced EAE. Figure 3A shows the disease scores of the wild-type and IFN-γR$^{-/-}$ mice sacrificed on day 8 (before clinical disease), day 14 (peak disease in wild-type mice) and day 20 (recovery in wild-type mice). Semiquantitative RT–PCR analysis detected very low levels of murine I-TAC in the CNS of IFN-γR$^{-/-}$ mice throughout the time course (lanes 1–9) while these mice developed increasingly

severe disease (Fig. 3B). Analysis of wild-type control mice, however, showed that I-TAC levels in the CNS were initially low on day 8 (lanes 1–3), were very high at the peak of disease when the mice begin to recover on day 14 (lanes 4–6), but then returned to low levels by day 20 (lanes 7–9) (Fig. 3C). These results do not provide a direct role for murine I-TAC during disease; however, they measure I-TAC expression during EAE for the first time.

DISCUSSION

The function and potency of human I-TAC suggests that it may be a key regulatory chemokine in the immune system. Previous studies suggest that antigen-presenting cells are an important source of this chemokine [6]. An analysis of murine I-TAC expression from DCs, which are key mediators of the immune response, however, has not been reported. To study the regulation of murine I-TAC in DC, we analysed its expression in two different DC progenitor cell lines, JAWS II and MTHC-D2, exposed to the cytokines IFN-γ, IL-4 and TNF-α. These analyses showed that murine I-TAC expression from DCs is regulated primarily by the IFN-γ signalling pathway and appears to be similar to the regulation of human I-TAC expression. Others have recently reported that IL-4 and/or TNF-α can also upregulate murine I-TAC in different cell types to those used here [22, 36]. Hence, it appears that the regulation of murine I-TAC can vary depending on the cell type and cytokine environment involved.

To further examine the regulation of murine I-TAC by cytokines and downstream signalling factors, we analysed the expression in Con A-activated splenocytes from a panel of knockout mice. These results confirmed the cell line data that murine I-TAC expression is regulated primarily by the IFN pathway. These assays also demonstrated that a proportion of I-TAC expression is dependent on the action of IRF-1, with the remainder probably being regulated by other IRF transcription factors involved in the interferon-signalling pathway [37]. The finding that murine I-TAC is in part regulated by IRF-1 may help explain some of the complex phenotypes of IRF-$1^{-/-}$ mice. IRF-$1^{-/-}$ mice have defective development of thymic $CD8^+$ cells, severe NK cell deficiency and an abnormally high proportion of Th2 $CD4^+$ T cells, which together lead to increased susceptibility to pathogens normally controlled by Th1 responses [37]. Thus the lowered expression of murine I-TAC from DCs in IRF-$1^{-/-}$ mice, combined with lowered Crg-2 and MIG expression (S Mahalingam, unpublished data), may cause the alteration in the balance of Th1/Th2 $CD4^+$ T cells.

Previously it has been shown that IFN-α or IFN-β enhance the expression of human I-TAC with or without other cell activation signals [3]. But, in our study, using the cells from various knockout mice, these two cytokines appeared to instead downregulate the level of murine I-TAC expression. However, during these analyses, the level of these cytokines in the cultures while not analysed, was probably low [38, 39]. Others have shown that low levels of IFN-α or IFN-β can increase the expression of IRF-2, which competes with IRF-1 thereby suppressing IRF-1-regulated genes [37]. Therefore in our assays, the lack of IFN-α/β signalling in the IFN-α/β$R^{-/-}$ and IFN-α/β-γ$R^{-/-}$ Con A cultures, and subsequent lack of IRF-2 expression, explains why murine I-TAC expression was higher in the wild type and IFN-γ$R^{-/-}$ Con A cultures, respectively. Thus, comparing our data with previously published findings, it appears that IFN-α and IFN-β can act both to increase or to decrease I-TAC expression depending on the relative amount of these two cytokines present [40].

Others have reported that TNF-α and IFN-γ synergize to enhance the expression of both murine and human I-TAC [7, 11, 22, 36]. We did not find any evidence of synergy between IFN-γ and TNF-α in either our cell line or Con A culture analyses. In our cell line assays, TNF-α was included primarily to promote cell differentiation during stimulation with other cytokines. Hence, in the analyses reported by others, TNF-α may have appeared to enhance the expression of I-TAC induced by IFN-γ through increased survival of differentiated cells. Others have also reported in a series of studies that for IP-10, a related chemokine, synergy between TNF-α/IFN-γ can be cell type and/or stimulus-specific [40]. Therefore, another explanation for our inability to detect synergy between these cytokines may be that our cell lines were different to those used in the previous reports and that Con A may not be an appropriate stimulus to observe the synergy in splenocyte cultures.

The addition of anti-CD40 Ab to MTHC-D2 cells showed that CD40 alone can upregulate the expression of murine I-TAC and that the CD40 and IFN-γ pathways can synergize to enhance this expression. This is consistent with a report by Altenburg *et al.* who showed that in cervical carcinoma cells, IFN-γ can synergize with CD40L to enhance the expression of a number of chemokines including IP-10, MCP-1, and to a lesser extent RANTES and MCP-3 [35]. Enhanced I-TAC expression, however, was not detected in the JAWS II cell line following CD40 activation. This result could be owing to differences in the level of CD40 expression between the two cell lines. The JAWS II cell line though lacks the tumour suppressor p53, and others have shown that p53-dependent functions can be regulated by CD40 [41]. Hence, the absence of IFN-γ/CD40 synergy in the JAWS II cell line could be explained by poor responsiveness in some of the downstream CD40 signalling pathways which involve p53. An alternative explanation is that the CD40 signalling pathway in the MTHC-D2 line was somehow enhanced owing to the unregulated expression of truncated c-Myb used to immortalize these cells [33]. In the Con A-activated $CD40^{-/-}$ splenocyte cultures, the lack of CD40 did not appear to reduce the murine I-TAC expression, which like the JAWS II cell line

suggests that CD40/IFN-γ do not synergize to enhance I-TAC expression. The absence of CD40 signalling, however, may have been compensated for by either direct costimulation of APCs by Con A, or more likely, by the large amounts of IFN-γ induced following Con A stimulation.

The APC/DC and T-cell interaction is frequently important in the regulation and exacerbation of autoimmune diseases. Given that I-TAC potentially enhances this interaction through the recruitment of activated T cells, we examined its role in an autoimmune disease model. Others have proposed that human I-TAC expressed in the CNS of MS patients may exacerbate the neuroinflammation associated with this disease [6, 8]. Our analysis of wild-type mice with MOG peptide-induced EAE demonstrated that murine I-TAC expression was the highest at the peak of disease severity, when the animals first began to recover. High expression at the peak of disease is consistent with a previous study that showed that known EAE-associated chemokines regulate the later CNS inflammatory processes as opposed to aiding the initial entry of autoreactive T cells into the CNS [15].

Recently, Willenborg *et al.* reported that IFN-γR$^{-/-}$ mice with MOG peptide-induced EAE develop severe disease and die, whereas wild-type mice develop mild disease and recover [13]. The report by Willenborg *et al.* demonstrated that IFN-γ, while primarily a proinflammatory cytokine, is also apparently important during disease recovery. In this same EAE disease model, we found that IFN-γR$^{-/-}$ mice expressed very low levels of murine I-TAC. Our results do not provide a direct link between the function of murine I-TAC and the EAE disease phenotype. It is, however, interesting to speculate, given the lack of murine I-TAC in the IFN-γR$^{-/-}$ mice, that I-TAC might have a role during disease recovery in wild-type mice by recruiting suppressor T cells. It is more likely, however, given that antibodies to a functional domain of CXCR3 protect mice from EAE, that murine I-TAC acts to enhance neuroinflammatory processes [42]. As there are other chemokines that bind to CXCR3, a thorough assessment of the contribution of murine I-TAC in the progression of neuroinflammation, however, remains to be performed.

In summary, murine I-TAC is a potent CXC chemokine and its expression is induced predominantly in response to IFN-γ. Our studies raise interesting questions as to the biological relevance of I-TAC since it is a chemokine that is expressed from progenitor DC lines and thought to recruit already activated CXCR3 expressing T cells. Murine I-TAC may therefore have an important role in DC/T cell interactions in early immune responses. This study should accelerate the thorough characterization of murine I-TAC and may have important implications for the development of vaccines and therapies for a range of different pathogens and diseases.

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APPENDIX 1C

Combinations of IFN-γ and IL-4 induce distinct profiles of dendritic cell associated immunoregulatory properties

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Running Title: DC-like characteristics induced by cytokine stimulus

Abstract

IFN-γ and IL-4 are necessary for the development of cell-mediated immunity (CMI) and humoral immunity (HI) respectively. How these cytokines differentially effect the development of DC immunoregulatory properties has not been resolved. To address this we have stimulated a DC progenitor-like cell line, MTHC-D2 with these cytokines. The expression of immunoregulatory molecules, phagocytic and T cell stimulatory function, and cellular morphology were differentially regulated. In addition, genetic profiling found IFN-γ induced expression of genes associated with mature DCs and important for CMI responses, whereas IL-4 induced genes associated with cellular adhesion and uptake of extracellular pathogens. Interestingly, the addition of both cytokines induced development of a unique set of immunoregulatory properties that were consistent with mature DCs but retained phagocytic properties characteristic of immature DCs. These studies demonstrate that IFN-γ and IL-4 can induce distinct immunoregulatory profiles that may underlie the ability of DCs to direct different adaptive immune responses.

Introduction

At the point of contact with a pathogen till the end of an adaptive immune response cytokines play an important role in directing and regulating immunity. Pathogens themselves can induce innate immune cells to secrete particular cytokines that go on to direct immune response development. Viruses for example, can induce IFN-γ production from NK cells that in turn stimulate DCs to induce IFN-γ-producing T cell responses important for CMI [1]. Whereas, yeast induces IL-4 production from DCs that supports HI [2]. How these cytokines differentially affect the immunoregulatory properties of DCs, that in turn allows them to direct these immune responses, is not well understood. One mechanism proposed is the regulation of DC IL-12 p70 production, where high levels induce CMI, and low levels support HI [3] [4] [5]. Given the association of IFN-γ with CMI, it would be expected that this cytokine could stimulate DCs to produce IL-12 p70. However, it has been shown that IFN-γ induces DCs to express the p40 homodimer form of IL-12 that instead antagonises p70's ability to induce CMI by competing for binding to the IL-12 receptor [6]. In the presence of IL-4, however, IFN-γ is able to induce high levels of p70 production from DCs [6] [7]. Therefore, a DC situated in a microenvironment containing both CMI and HI associated cytokines is more able to support IL-12 p70 production. It is more likely the DC exposed to IFN-γ utilises other, as yet unidentified mechanisms to induce CMI as recent studies have shown that DCs can induce this type of immunity in the absence IL-12 p70 [8]. Detailed analysis of the types of immunoregulatory molecules induced within DCs in response to cytokine combinations of IFN-γ and IL-4 may provide insight into how CMI and HI cytokine microenvironments influence the DCs ability to support different types of adaptive immunity.

To examine cytokine induced changes to the immunoregulatory properties of DCs it is beneficial to utilise DC progenitor or immature cells because the maturation pathway is uncommitted, and they are thought to reside in and/or enter infected tissues early during an immune response [9]. Cell lines that mimic progenitor DCs are well suited to such studies as they provide large numbers of homogeneous cells for molecular examination that requires a high degree of consistency. To confirm the cell line findings results from these analyses can then be cross-compared with DCs isolated *ex vivo* that have been exposed to inflammatory and HI responses. A cell line that mimics progenitor DCs is the cloned myb-transformed hematopoetic cell line, MTHC-D2, derived from C3HeJ mouse fetal liver cells [10] [1] [11]. MTHC-D2 has myeloid precursor cell properties and like myeloid DCs, is GM-CSF dependent. This line expresses F4/80, Mac-1, FcγRII, Gr-1, and Pgp-1 markers, does not express T and B lymphocyte antigens, is esterase negative and functionally is weakly pinocytotic and non-phagocytic [10]. MTHC-D2 cells have been previously shown to differentiate into cells with DC-like properties, including the expression of DC associated

markers, in response to effector cytokine stimulation [10] [1].

In this study we report the cellular and functional changes of MTHC-D2 following stimulation with IFN-γ and IL-4 separately or together. TNF-α was also used to stimulate the cells in combination with IFN-γ and IL-4 as this cytokine is produced at early stages of an immune response, and has been shown to induce morphological differentiation of MTHC cells and mediate myeloid cell maturation [12] [13] [11]. The morphology, cell surface marker phenotype, gene expression profile, phagocytic activity and ability to stimulate T cell proliferation were all examined.

Materials and Methods

Maturation and activation of the MTHC-D2 cell line

MTHC-D2 cells were cloned previously from the MTHC myb-transformed progenitor cell line derived from C3H.HeJ murine fetal liver cells [14] [11]. The cells were maintained in Iscove's modified eagle medium (IMEM; GIBCO BRL) supplemented with 10% FCS, 2 mM glutamine (SIGMA), 60 U/mL of gentamicin (SIGMA), and 50 U/mL GM-CSF. MTHC-D2 cell maturation was induced by exposure for 6 h (cDNA subtraction analysis) or 48 h (all other analyses) to TNF-α (500 U/mL) and GM-CSF (5 U/mL), and either IFN-γ (2 U/mL) or IL-4 (50 U/mL), or both IFN-γ plus IL-4. Sequential addition of IFN-γ and IL-4 was achieved by adding the one of these cytokines 24 h after the other cytokine. This resulted in transdifferentiation of MTHC-D2 cells that was observed morphologically within 6 h when IL-4 was added to IFN-γ exposed cells or when IFN-γ was added to IL-4 exposed cells. CD40 Ab purified from FGK45.5 [15] was used at 25 μg/mL to activate maturing MTHC-D2 cell types by CD40 cross linking for the final 16 h of differentiation.

Transmission electron microscopy (TEM)

Cytokine-stimulated and un-stimulated MTHC-D2 cells ($5X10^6$ to 10^7) were washed once with PBS then fixed with 2% glutaraldehyde (LADD Research Industries, Inc, Vermont, USA) in 0.1 M sodium cacodylate (BDH) buffer pH 7.4 for 2 h, followed by two 30 min washes in fresh PBS. Secondary fixation was carried out using 1 % osmium tetroxide (BDH) in 0.1 M sodium cacodylate buffer pH 7.4 for 1.5 h followed by two 30 min PBS washes. Samples were washed a further three times in distilled water, stained *en bloc* in 2 % uranyl acetate (BDH) for 2 h and rinsed another three times in distilled water. Samples were

dehydrated in graded alcohol series, embedded in Spurrs' Resin (Agar Scientific, Essex, UK) and sectioned to 80 nm using a Reichhert Ultracut E (Austria). Sections were then stained with Reynold's lead citrate (BDH) for 15 min and examined using a Phillips EM 301 (The Netherlands) electron microscope at 60 kV (JCSMR EM facility).

FACS analysis of immunofluorescently labeled MTHC-D2 cells

Commercially available antibodies were used to stain cytokine-stimulated and un-stimulated MTHC-D2 cells ($1\text{-}5x10^{5)}$ according to the manufacturer's instructions and analysed using a Becton-Dickinson bench-top Flow Cytometer (FACScan) using the standard filter configuration (JCSMR FACS sorting facility). Samples were prepared in triplicate and experiments were repeated. Data were analysed using Becton-Dickinson Cell Quest^{TM} software and an experiment representative of those performed is presented. The average of triplicate medium relative fluorescent intensities of unstained controls and stained samples and their standard deviation are presented. Commercial antibodies used included CD11c (Pharmingen), CD69 (Pharmingen), CD90 (CALTAG), CD11b (CALTAG), CD45R (B220) (CALTAG), CD8α (Pharmingen), CD4 (Pharmingen), CD44 (Pharmingen), CD40 (Southern Biotechnology Associates, Inc), CD80 (Pharmingen), CD86 (Pharmingen), CD8β (Pharmingen), and DEC-205 (Serotec).

Phagocytosis Assays

Cytokine-stimulated and un-stimulated MTHC-D2 cells were co-cultured with haemolysin sensitised sheep red blood cells (SRBC) for 1 h at 37 ^{o}C and then stained with May-Grunwald and Giemsa dyes and visualised by light microscopy, or processed as described above for TEM. The percentage of MTHC-D2 cells undergoing phagocytosis, and the number of phagocytosed SRBCs per cell, were determined by light microscopy by the analysis of 50 randomly selected cells in each sample.

Mixed Lymphocyte Reactions (MLRs)

MLR reactions were performed using a cell proliferation assay that enables proliferation of particular cell types to be monitored simultaneously by FACS analysis of CFSE-dye labeled cells [16]. Briefly, the cell division of cytokine plus CD40Ab stimulated and un-stimulated MTHC-D2 cell samples ($5 \text{ X } 10^{7}$ cells/mL) was blocked with 50 µg/mL Mitomycin C (Sigma) for 20 min, 37^{o}C in the dark and washed four times with media.

Splenocytes were prepared from six week old female BALB/c mice by passing spleens through a sieve and lysing the red blood cells using 5 mL Tris NH_4Cl / spleen as well as wool column purification to remove adherent monocyte cells. Splenocytes were then loaded with CFSE dye (10 μM CFSE in 4 mL media) (Molecular Probes) for 8 min at room temperature then washed three times with fresh media. The ratio of stimulator MTHC-D2 cells to splenocytes in MLRs was 1:40. Cells were co-cultured for seven days at 37°C. T cell proliferation was monitored periodically over the culture period by FACS detecting the sequential loss of CFSE dye in proliferating CD90 (Cy-chrome-conjugated, Caltag), CD8α (Cy-Chrome-conjugated, Pharmingen) or CD4 (PE-conjugated, Pharmingen) positively stained cells. 5470 fluorospheres (Coulter Corporation) were added to each sample to enable accurate counting of proliferating cells and cross comparison between MLR samples. Data was analysed using Becton-Dickinson Cell Quest™ software and experiments representative of those performed were presented as the number of positively stained proliferating cells on a given day of the MLR.

cDNA subtraction libraries

Four cDNA subtracted libraries were prepared comparing MTHC-D2 cells cultured in GM-CSF, with MTHC-D2 cells stimulated for 6 h by the addition of either IFN-γ or IL-4 in the presence of TNF-α and GM-CSF. mRNA (2 μg) was extracted from the different cell preparations (QuickPrep Micro mRNA purification system, Pharmacia Biotech) and used for cDNA subtraction (PCR-Select cDNA subtraction kit; Clontech, USA). Plasmid libraries were prepared from subtracted cDNAs using the UDG pAMP10 cloning system (GIBCO BRL). One to two thousand plasmid clones from each subtracted library were screened for differentially expressed cDNAs (PCR-Select Differential Screening Kit, Clontech, USA). The differentially expressed cDNA clones were sequenced using Big Dye Terminator automated sequencing (ABI sequencer, Biomolecular Resource Facility, JCSMR). Nucleotide sequences were screened against nucleotide databases at NCBI using the BLASTn programme. Selected cDNA sequences with homology to known genes were sorted into gene categories.

Northern analysis of selected differentially expressed cDNAs

Isolated mRNA (2 μg) from cytokine-stimulated and un-stimulated MTHC-D2 cell samples were separated by formaldehyde agarose gel electrophoresis, transferred onto nylon membrane (BrightStar-Plus, Ambion) and probed with ^{32}P-labeled cDNA from selected plasmid clones (Strip-EZ DNA system, Ambion). Positively hybridised bands on northerns

were quantified on a PhosphorImager using ImageQuant software. Band intensity values for each northern were normalised to murine GAPDH and scaled such that the highest band value on all northerns was 10.

Semiquantitative RT-PCR analysis of IL-12 p40, p35, IL-10, and IFN-γ/ IL-4 induced genes

Isolated mRNA (2 μg) (QuickPrep Micro mRNA purification system, Pharmacia/Biotech) from cytokine plus CD40Ab-stimulated and un-stimulated MTHC-D2 cell samples was used to prepare first strand cDNA (Ready-To-Go cDNA synthesis kit, Amersham, Pharmacia, Biotech, Inc). Expression of MMR, crg-2, IL-10, I-TAC, MHC class II, IL-12 p35 and p40 were standardised against GAPDH expression using hot-start semi-quantitative RT-PCR analysis. PCR parameters were optimised to insure the PCR products obtained did not proceed past the linear phase of the reaction that would exceed the analysis limits. Experimental findings were repeated three times. Primer pairs and resulting fragments sizes were as follows: MMR AGACGAAATCCCTGCTACTG / CTGGACTCAGCAGCAG TCTC 195 bp, crg-2 ATGAACCCAAGTGCTGCCGTC / CTTGAGTGAGGACTCAGACC 538 bp, IL-10 TAGAGCTGCGGACTGCCTTCA / TCATGGCCTTGTAGACACCTTG 350 bp, I-TAC GAACAGGAAGGTCACAGCCATAGC / ATGAGGCGAGCCTGCTTGGATC TG, MHC class II AGTCTTCCCAGCCTTCACCTCAGAGGTAC / CATAGCCCCAAAT GTCTGACCTCTGGAGAG 250 bp, IL-12 p35 GGCTACTAGAGAGACTTCTTCC / GT GAAGCAGGATGCAGAGCTTC 309 bp, IL-12 p40 CGTGCTCATGGCTGGTGCAAAG / CTTCATCTGCAAGTTCTTGGGC 313 bp and GAPDH TCCACCACCCTGTTGCTGTA and ACCACAGTCCATGCCATC AC 432 bp. Advantage-2 polymerase (0.5units)(Clontech, USA) was added at 94°C (hot start). I-TAC and GAPDH were amplified for 30 cycles whereas IL-10, IL-12 p35 and IL-12 p40 were amplified for 34 cycles using the thermal program: 94°C 20 sec, 64 °C 20 sec, 72 °C 30 sec. MHC class II was amplified for 32 cycles using 94°C 20 sec, 60 °C 20 sec, 72 °C 20 sec. MMR and crg-2 were amplified for 33 cycles of 94°C 20 sec, 62 °C 20 sec, 72 °C 20 sec.

Results

Morphological analysis of cytokine and CD40 Ab stimulated MTHC-D2 cells

During maturation of DCs from progenitor cells a morphological change is evident. Progenitor cells are round and non-adherent whereas mature DCs develop longer dendrites, become adherent, and are often described as irregular or star shaped [9]. To examine the

morphological changes induced by cytokine exposure, the MTHC-D2 cells were stimulated for 48 h, then examined by light microscopy that detects shape and adherence state (data not shown), and transmission electron microscopy (TEM) that detects intracellular changes (Fig. 1). As TEM required processing of the cells in a non-adherent state the shape differences were not as evident. The morphology of un-stimulated GM-CSF cultured cells was most like the phenotype of progenitor myeloid cells being round, and non-adherent by light microscopy analysis, and having a large irregular nucleus (Fig. 1A). The IFN-γ+TNF-α stimulated cells tended to develop a morphology similar to mature DCs being irregular or star shaped with longer dendrites when assessed by light microscopy. TEM analysis however, found the intracellular structure of these cells to be similar to the progenitor GM-CSF cultured cells although exosome release, a property of *ex vivo* DC's [17], was visible at the cell surface (Fig. 1B). The IL-4+TNF-α cultured cells appeared more immature, with many cells remaining non-adherent, and TEM detected an assortment of larger intracellular vacuoles (Fig. 1C) not evident in the IFN-γ+TNF-α or un-stimulated cells. The cytokine combination IFN-γ+IL-4+TNF-α induced dramatic changes to both the intracellular and extracellular morphology of the MTHC-D2 cells. Similar to mature DCs these cells became adherent and developed longer dendrites. These cells were different however, to the other cytokine stimulated MTHC-D2 cells being generally larger, round and contained a substantially greater number of intracellular vacuoles (Fig. 1 D). Interestingly these vacuoles were often found to contain phagocytosed cell debris or apoptotic bodies. Surprisingly, this morphology could also be obtained rapidly, within 6 h, by the sequential addition of IL-4 to IFN-γ+TNF-α stimulated cells or IFN-γ to IL-4+TNF-α stimulated cells, suggesting the cells were capable of transdifferentiation. Further experiments using a CD40 Ab co-stimulus found that, like *ex vivo* DCs, this stimulus enhanced the maturation state by increasing cellular adherence.

Cytokine stimulus and CD40 activation of the MTHC-D2 cells differentially regulates the expression of cell surface markers associated with DC maturation and subtype

The maturation and activation state of DCs affects their ability to stimulate T cells and is often characterised by differences in the expression levels of key cell surface markers. CD40 co-stimulation of DC's usually enhances the maturation and activation state of the cells as indicated by increased expression of these markers. In this study, the cytokine and activation stimulus were found to induce different expression patterns of these cell surface markers in the MTHC-D2 cells, suggesting various levels of cellular maturation were obtained. Similar to progenitor DCs [9], little expression of co-stimulatory molecules CD40, CD80 and CD86 were detected on the GM-CSF un-stimulated cells and CD40 activation did

not enhance the expression of these molecules (Table 1). Like mature *ex vivo* DCs, exposure to IFN-γ+TNF-α and IFN-γ+IL-4+TNF-α increased expression of these costimulatory molecules and this was further enhanced by exposure to CD40 Ab. Activation of these cells was evident by increased expression of the activation marker CD69. IL-4+TNF-α also induced the MTHC-D2 cells to express CD40, CD80 and CD69 but unlike the cells exposed to IFN-γ, these cells had a lower level of CD86 expression, and CD40 activation did not enhance the expression of these molecules.

DC-subtype and macrophage markers CD8α, CD11c, CD11b, DEC-205, and CD44 are frequently used to characterise cells with DC morphology. These markers were also differentially induced in response to the combinations of cytokine stimulation. In particular, the combined IFN-γ+IL-4+TNF-α stimulus had the greatest effect on the expression of most of these molecules, except for CD8α expression where IL-4+TNF-α also induced high levels of expression of this molecule. The effect of CD40 co-stimulation on the expression of these molecules was more variable in the IFN-γ+TNF-α and IFN-γ+IL-4+TNF-α stimulated cells compared with the co-stimulatory/activation markers, and once again had no effect on the IL-4+TNF-α stimulated cells suggesting a CD40 signaling block.

Other markers including CD4, a DC subtyping marker, CD8β, a marker of a specific DC subset in rats, B cell marker CD45R, and the T cell marker CD90.2, showed very low levels of expression in all MTHC-D2 cells (data not shown).

The level of phagocytic activity directly corresponds with the type of cytokine stimulus of MTHC-D2 cells

The phagocytic potential of DCs usually changes during maturation where cells are generally more phagocytic in an immature state and, as they mature and become activated, the level of phagocytic activity declines and the cell directs antigen for presentation on the cell surface [9]. However, there are now some reports of mature cross-presenting DCs that are highly phagocytic at the site of inflammation [18]. To establish whether cytokines differentially regulate phagocytosis, Fc receptor mediated uptake of SRBC's was examined for each cytokine stimulated MTHC-D2 cell type. Intracellular staining, visualised by light microscopy was used to establish the percentage of MTHC-D2 cells undergoing phagocytosis and estimate the number of SRBCs within each cell (Fig. 2). Substantial differences in phagocytic activity were found in association with the cytokine induced maturation/activation state of these cells. This analysis found the IFN-γ+IL-4+TNF-α stimulated MTHC-D2 cells were twice as phagocytic as the cells stimulated with IL-4+TNF-α, followed by the IFN-γ+TNF-α, and the un-stimulated GM-CSF cultured cells. CD40 co-stimulation, which enhances

maturation of DC's, down-regulated the level of phagocytosis of the IL-4+TNF-α and IFN-γ+TNF-α stimulated cells. This however, had no effect on the IFN-γ+IL-4+TNF-α stimulated cells suggesting these cells, like mature cross-presenting DCs, retain their phagocytic ability in a mature state.

TEM enabled detailed analysis of the intracellular structure of the MTHC-D2 cells undergoing phagocytosis (Fig. 3). The morphology of the GM-CSF un-stimulated cells, that had little phagocytic activity, were not affected by the presence of SRBC's after 1 h of co-culturing (Fig. 3aA). However, the presence of SRBC's caused the IFN-γ, IL-4 and TNF-α stimulated DCs to extend the length of their dendritic protrusions (Fig. 3aB,C,D) compared with these cell types in Figure 1. Often these protrusions were found to be reaching out for, and encompassing, the SRBC's (Fig. 3aC,D). Interestingly, those cells degrading the SRBC's had relatively smooth cell surfaces with shorter dendritic protrusions (Fig. 3b). These observations support the findings of phagocytic activity observed by light microscopy, and indicate the presence of extracellular antigen (SRBC's) induces these cells to alter their morphology to enable engulfment of the antigen. These changes are supported by other studies where the surface area of phagocytic cells increased [19], and the surface folds were found to disappear during phagocytosis [20].

IL-4 and IFN-γ differentially affect the ability of MTHC-D2 cells to stimulate mixed lymphocyte reactions (MLR)

One of the most important features of a DC that supports the development of adaptive immunity is the ability to stimulate T cell proliferation [9]. This function distinguishes DCs from other APC's such as macrophages. The ability of a DC to induce T cell proliferation is also associated with maturation/activated state of the cell, where immature DCs are unable to stimulate high levels of T cell proliferation [9]. To assess whether particular cytokines affect the ability of MTHC-D2 cells to stimulate T cell proliferation, MLR's were performed and analysed using a CFSE cell proliferation FACS assay [16]. This approach enabled the proliferation of both CD4 and CD8 T cells to be monitored simultaneously, a procedure that the traditional (^{3}H)-Thymidine uptake MLR assay is unable to do.

In this study, initially MLR's were performed using cytokine stimulated MTHC-D2 cells (not activated by CD40 Ab cross-linking), and proliferating T cells were monitored with the CD90 antibody to detect both CD4 and CD8 T cells (Fig. 4, MLR 1). This analysis found the IFN-γ+TNF-α stimulated MTHC-D2 cells induced the highest level of T cell proliferation, followed by IFN-γ+IL-4+TNF-α, IL-4+TNF-α, and un-stimulated GM-CSF cultured MTHC-D2 cells. When these cells were activated with CD40 Ab and assessed for

the ability to stimulate T cell proliferation using CD8 and CD4 specific antibodies (Fig.4, MLR 2, MLR 3), these cells induced T cell proliferation faster (1 day earlier) than the un-activated cells. This response is similar to the effects of CD40 activation on the T cell stimulatory capacity of *ex vivo* DCs. Interestingly, each MTHC-D2 cell type induced a greater proportion of CD8 (Fig.4, MLR 2) compared with CD4 (Fig.4, MLR 3) T cell proliferation. This result was not un-expected as induction of CD8 T cell proliferation is a common feature of MLR's [9]. The IFN-γ+TNF-α stimulated MTHC-D2 cells were the most potent at stimulating CD8 T cells, followed by the IFN-γ+IL-4+TNF-α stimulated cells. Whereas, the IL-4+TNF-α stimulated and un-stimulated GM-CSF cultured cells were weak inducers of CD8 T cell proliferation. Both IFN-γ+TNF-α and IFN-γ+IL-4+TNF-α stimulated MTHC-D2 cells induced CD4 T cell proliferation, whereas the IL-4+TNF-α and un-stimulated GM-CSF cultured MTHC-D2 cells were weak inducers of CD4 T cells. These results are similar to studies of monocyte derived DCs, where the best stimulators of T cell proliferation had the highest levels of maturation, and this was further enhanced by CD40 co-stimulation [9] [21]. These results also suggest that, as the IFN-γ and IL-4 cytokine microenvironment *in vivo* changes, it is likely to affect the ability of DCs to stimulate T cell proliferation.

Genes induced by IFN-γ+TNF-α and IL-4+TNF-α stimulation of MTHC-D2 cells

Biological analysis of the MTHC-D2 cells morphology, cell surface marker phenotype, phagocytic ability, and T cell stimulatory capacity, identified distinct changes to maturation, activation and functional state of these cells in association with specific cytokines and CD40 co-stimulation. To examine whether particular gene expression profiles were also associated with specific cytokine stimulation, molecular analysis using cDNA subtraction was performed. This analysis targeted genes that were induced early (6 h) after cytokine stimulation. Four cDNA subtraction libraries were prepared, including 1 IFN-γ+TNF-α subtracted from IL-4+TNF-α stimulated MTHC-D2 cells; 2 IL-4+TNF-α subtracted from IFN-γ+TNF-α stimulated MTHC-D2 cells; 3 un-stimulated GM-CSF subtracted from IFN-γ+TNF-α stimulated MTHC-D2 cells; 4 un-stimulated GM-CSF subtracted from IL-4+TNF-α stimulated MTHC-D2 cells. Differentially expressed cDNA clones from these libraries were sequenced and screened against the NCBI nucleotide database using a BLASTn programme. Those sequences sharing homology of greater than 95% over more than 100 bp with known gene sequences were sorted into gene categories. Selected cDNAs having homology to genes with an immune function, or association with DCs and macrophages, as well as the frequency of these clones in each library, are listed in Table 2. To establish the level of differential expression, some of these clones were also screened by northern analysis of mRNA from the cytokine stimulated MTHC-D2 cells (Table 2). This comprehensive study

enabled a genetic profile to be developed that identifies unique and common features between IFN-γ+TNF-α verses IL-4+TNF-α stimulation of the MTHC-D2 cells. Overall the cytokine stimulus affected the expression of genes involved in many levels of cellular organisation and function including signaling, transcription and cytoskeletal genes, cell surface receptors, secreted molecules, as well as genes associated with cell migration and immune responses.

The profile of genes from IFN-γ+TNF-α specific libraries (2 and 3) suggested a more mature DC, with anti-viral and CMI potential. This included a range of chemoattractants such as MIG, crg-2, I-TAC, and RANTES that attract activated and memory Th1 cells [22] [23]. Also, Cathepsin C was identified, which is required for activation of granzymes and is thought to play a role in the expansion and differentiation of CD8 T cell cytolytic activity [24] [25]. Mouse fibrinogen-like protein, which is up regulated in response to mouse hepatitis strain 3 infection [26] was also identified. In addition, IFN-γ inducible protein Mag-1 was up regulated and has been shown to accompany an increase in Th1 cytokine expression in mice infected with *Toxoplasma Gondi* and undergoing progressive encephalitis [27]. IFN-γ+TNF-α stimulation also induced expression of activation marker CD69, as was detected by FACS analysis, and MHC II, which is a marker of mature DCs and is important for antigen presentation. Transcription factor IFN-inducible Sp100, whose function in early antiviral responses has been postulated [28], was also identified. Signaling genes such as numerous types of GTP and GBP binding proteins were specifically expressed in response to IFN-γ+TNF-α, as well as interferon inducible IFN regulatory factor 1 and 7 genes. Interestingly, Mx1, a gene associated with inhibiting influenza virus replication [29] [30], contains a GTP binding domain that is essential for its anti-influenza activity [30], suggesting the importance of GTP molecules in anti-viral immune responses.

Genes from the IL-4+TNF-α specific libraries (1 and 4) were associated with receptor-mediated endocytosis of pathogens or tumor cells, such as the macrophage mannose receptor (MMR), Mg1 receptor, and FcγR. In addition, an assortment of genes associated with cytoskeletal restructuring and lysosomal processing of antigen, such as gelsolin, cytoplasmic gamma actin, and cathepsin B, L, G identified. Expression of these genes may be responsible for the increased number of intracellular vacuoles detected by TEM of the IL-4+TNF-α MTHC-D2 cells and may also be associated with the enhanced phagocytic function of these cells. Also of interest, was the absence of MHC II in the IL-4+TNF−α specific libraries but not the IFN-γ+TNF-α specific libraries, as IL-4 is routinely used to induce expression of this gene in maturing monocyte derived *ex vivo* DCs [31] [32]. These results suggest that instead, IFN-γ or another stimulus may be responsible for expression of MHC II in the *ex vivo* DCs.

Some cDNA clones were identified in libraries that compared the IFN-γ+TNF-α and IL-4+TNF-α stimulated with the un-stimulated GM-CSF cultured MTHC-D2 cells (3 and 4).

This suggests in CMI and HI cytokine microenvironments that DCs may have particular cellular activities in common. These included genes associated with DC adhesion (ICAM-1 and ryudocan core protein), antigen processing and presentation (TAP, B_2M and MHC I), as well as T cell co-stimulation (CD40). Northern analysis of these cDNA's however, found them to be induced to different levels by the cytokine stimulus, such as CD40 and ICAM-1 were expressed at much higher levels in the IFN-γ+TNF-α stimulated cells, whereas MHC I was expressed at higher levels in the IL-4+TNF-α stimulated cells. The differential expression of these molecules in various cytokine microenvironments may affect the efficiency of DCs to perform specific immunoregulatory tasks.

Comparison of the northern results detected a trend in the expression patterns of cDNAs isolated from IFN-γ+TNF-α and IL-4+TNF-α specific libraries (Table 2). Generally cDNAs isolated from the IFN-γ+TNF-α libraries were expressed 7 fold higher (using an average of 12 selected cDNAs) in the IFN-γ+TNF-α cells when compared to the other MTHC-D2 cell types by northern analysis. Whereas, the cDNAs isolated from the IL-4+TNF-α specific libraries were often also quite highly expressed by northern analysis in other MTHC-D2 cell types, having only 4 fold greater expression (using an average of 17 selected cDNAs). This suggests that many of the genes induced by IFN-γ+TNF-α are uniquely important to the function of these cells in this cytokine microenvironment, whereas those genes induced by IL-4+TNF-α may also be important to the function of the cell in other cytokine microenvironments. For example, the cytokine TGF-β, the Mg1 receptor, FcγR, and the MAC-2 antigen, are expressed at high levels on both the un-stimulated GM-CSF cultured, and IL-4+TNF-α stimulated MTHC-D2 cells. This suggests that DCs, in the absence of IFN-γ, have properties important for the uptake of extracellular pathogens (FcγRII) and tumor cells (Mg1), antibody mediated endocytosis (MAC-2), and by the action of TGF-β, suppress cytotoxic T cells, influence the activation and differentiation of T cells, and the expression of co-stimulatory molecules [33] [34] .

Effects of combining IFN-γ and IL-4 stimulation on the expression of DC immunoregulatory genes

Combining the effector cytokine stimuli (IFN-γ+IL-4) found substantial differential effects on the morphology, cell surface marker phenotype, as well as phagocytic and T cell stimulatory activity of the MTHC-D2 cells, compared to stimulation with these cytokines independently. Whether these cytokines induce a distinct genetic profile, as was found for these cytokines independently by cDNA subtraction, requires additional genetic analysis. Of the limited number of studies that have examined the combined effect of these cytokines on

DCs they have been shown to be more effective at inducing production of the CMI promoting bioactive form of IL-12 (p70) [5]. We therefore examined the effect of combining these cytokine stimuli on expression of the IL-12 p40 and p35 gene subunits, as well as IL-10, a regulator of IL-12 production [35] [36]. We also examined the combined effect of IFN-γ+IL-4 on genes that were induced by these cytokines independently in cDNA subtraction, including MHC II, crg-2, I-TAC, and MMR (Fig. 5).

In this study, IL-12 p40 was differentially regulated by cytokine and CD40 Ab stimulation of the MTHC-D2 cells (Fig. 5). Unlike IL-4+TNF-α, IFN-γ+TNF-α stimulation induced p40 expression. Combining IL-4+IFN-γ+TNF-α stimulation also induced p40 expression but not as strongly as IFN-γ+TNF-α, suggesting the IL-4 in this cytokine combination had an inhibiting effect. CD40 co-stimulation enhanced p40 gene expression, especially in the un-stimulated GM-CSF cultured MTHC-D2 cells and those stimulated with IL-4+IFN-γ+TNF-α. In these later cells, CD40 co-stimulation appeared to compensate for the inhibiting effect of IL-4. The effect of CD40 co-stimulation was not as strong in those cells stimulated with IFN-γ+TNF–α, where a two-fold increase was detected. The expression of p40 is similarly regulated in *ex vivo* DCs, where IL-4 down regulates, and IFN-γ up regulates expression [37] [4], and combining these cytokine stimuli decreases p40 expression compared with IFN-γ stimulation alone [6]. In *ex vivo* DC's IFN-γ induces the p40 homodimer form of IL-12 p40 that antagonises p70-induced CMI by competing for the IL-12 receptor, whereas IFN-γ+IL-4 stimulation induces the p40 monomer that supports bioactive IL-12 p70 production [38] [39] [40] [6]. The studies on MTHC-D2 suggest that *in vivo* bioactive IL-12 production in an IFN-γ+IL-4 cytokine microenvironment requires CD40 co-stimulation. The requirement for T cell-derived or microbial stimulus to induce IL-12 production has also been reported for *ex vivo* DCs [6]. Unlike p40, p35 expression was detected in all cells, and the level of expression was not as effected by the different combinations of cytokine and CD40 co-stimulation. Similar to p40 however, as well as studies on *ex vivo* DCs [41], p35 was up regulated marginally by IFN-γ compared with the un-stimulated and IL-4+TNF-α stimulated cells. In addition, p35 expression was up regulated by CD40 co-stimulation in cells stimulated with cytokine combinations containing IL-4, whereas co-stimulation had little effect on the IFN-γ+TNF-α stimulated cells.

IL-10 expression in the MTHC-D2 cells was also examined by RT-PCR as it is a down-regulator of bioactive IL-12 p70 production [35] [36], and important for the development of HI [42]. This analysis found the greatest levels of IL-10 expression in association with IFN-γ+TNF-α stimulation, a feature that may contribute to the inability of IFN-γ to induce high levels of IL-12 p70 production. Lower levels of IL-10 expression however, were associated with IFN-γ+IL-4+TNF-α and CD40 Ab stimulation, which likewise

may contribute to the ability of these stimulants to induce high levels of IL-12 p70 production. Interestingly, a similar level of IL-10 expression was associated with un-stimulated GM-CSF cultured cells that had been activated with CD40 Ab. This suggests DCs, in the absence of IFN-γ and IL-4, but exposed to CD40 co-stimulation, may also support IL-12 p70 production. In support of this, one study has found that immature DCs stimulated with CD40 L or bacteria stimulate more IL-12 p70 production than mature DCs [43].

MHC II is an important molecule enabling DCs to present antigen to T cells and is often used as an indicator of the mature state of DCs [9]. In our study, cDNA subtraction identified greater levels of expression of this gene in cells stimulated with IFN-γ+TNF-α compared with IL-4+TNF-α stimulated cells (Table 2). RT-PCR analysis detected similar results, and found combining these cytokine stimuli also induced MHC II but not as strongly as IFN-γ+TNF-α stimulation. Activation with CD40 Ab, which enhanced the level of many mature DC associated markers in the MTHC-D2 cells (Table 1), also enhanced the level of MHC II expression in both the IFN-γ+TNF-α and IFN-γ+IL-4+TNF-α stimulated cells.

The chemokines crg-2 and I-TAC, are important for attracting T cells in CMI and were found to be up regulated by IFN-γ in the cDNA subtraction studies reported here, and by others [44] [45] [23]. Similarly to the effects of combining IL-4 and IFN-γ stimulus on the expression of MHC II, p40, p35 and IL-10, these cytokines also reduced the level of expression of crg-2 and I-TAC in the MTHC-D2 cells. Interestingly, CD40 activation enhanced the level of I-TAC expression but reduced crg-2 expression, suggesting these chemokines may be differentially regulated during DC/T cell interactions.

The MMR is a phagocytic receptor for microorganisms and is known to be down regulated by IFN-γ [46]. In our study, RT-PCR analysis confirmed the effect of IFN-γ on this gene, as well as the cDNA subtraction data showing that IL-4+TNF-α induces greater levels of expression of this gene compared with IFN-γ+TNF-α. Combining these cytokine stimuli induced slightly lower levels of expression compared with IL-4+TNF-α stimulation suggesting that IFN-γ does have an inhibitory effect in this cytokine combination. CD40 co-stimulation, that effected the expression of other immunoregulatory genes, did not appear to effect the expression of MMR except for the IFN-γ+TNF-α stimulated cells where reduced expression was detected. By comparison to the effects of IL-4 on the IFN-γ-induced genes, the effect of IFN-γ on the MMR is marginal, which confirms the observation made by northern analysis (Table 2), that IL-4 induced genes are often also expressed at similar levels in response to other cytokine stimuli and therefore may be important to the function of the cell in various cytokine microenvironments.

Discussion

This study has examined whether CMI and HI associated cytokines regulate the development of particular DC associated immunoregulatory properties. This study established that IFN-γ+TNF-α can induce the MTHC-D2 cell line to develop characteristics common of mature DCs as well as immunoregulatory properties that would likely be supportive of anti-viral responses and CMI. This was demonstrated by increased levels of co-stimulatory and MHC molecules, increased capacity to stimulate T cell proliferation, as well as IFN-γ induced expression of genes associated with inhibiting viral replication and that support cytolytic activity of CD8 T cells. Expression of these types of CMI associated genes may contribute to the IFN-γ stimulated DC's ability to induce CMI in the absence of IL-12 p70.

Combining IFN-γ+IL-4+TNF-α stimulation also induced the MTHC-D2 cells to develop immunoregulatory properties associated with mature DC's. However, compared with IFN-γ+TNF-α stimulated cells, genes induced by IFN-γ stimulation, that would support CMI, such as I-TAC and crg-2, were down regulated, whereas properties associated with the up take of extracellular pathogens, such as phagocytosis and expression of MMR, that were induced by IL-4 stimulation, were up regulated in these cells and retained after CD40 co-stimulation. Combining these cytokine stimuli also reduced the ability of the cells to stimulate CD8 T cell proliferation compared with IFN-γ+TNF-α stimulation, an effect that has also been detected in *ex vivo* DCs [47]. Although this combination of cytokines induces bioactive IL-12, that supports CMI, our study suggests these cytokines may induce the development of other immunoregulatory properties that also support HI. Taken together, this cell type may actually facilitate the progressive change between these types of immunity.

Although DC co-stimulatory molecules and T cell stimulatory capacity were induced upon maturation of MTHC-D2 cells by IL-4+TNF-α compared to un-stimulated cells, it was less than that obtained with IFN-γ+TNF-α and IFN-γ+IL-4+TNF-α stimulation. It is possible the IL-4+TNF-α MTHC-D2 cells have not as yet reached full maturity especially as studies on a CD14+ DC-like cell line, XS52 [48], and monocyte derived *ex vivo* DCs [31], require seven to eleven days to mature these cells with IL-4. Alternatively, the IL-4+TNF-α stimulated cells may represent a macrophage although this is unlikely as these cells were unable to produce esterase [10], and IL-4 suppresses differentiation along the macrophage pathway [49]. The most likely possibility, based on the data, is that the IL-4+TNF-α cells have developed properties that support HI development. For example, by 48 h of IL-4+TNF-α stimulation the MTHC-D2 cells had enhanced their ability to take up extracellular antigen, as demonstrated by increased phagocytic activity, expression of pattern recognition receptors,

such as FcγRII and the MMR [50] [51], as well as increased expression of cytoskeletal genes and increased numbers of intracellular vacuoles that support phagocytosis [52] [50].

Our studies have detected a number of correlations between *ex vivo* splenic DCs and the MTHC-D2 cells in relation to cytokine stimulation, expression of DC sub-typing markers CD8α, CD4, and DEC-205, as well as IL-12 p40 expression (Table 3). *Ex vivo* DCs, that produce the greatest amounts of IL-12 p70, are either CD8α+ splenic DCs [53], or $CD11c^{+}$ splenic DCs stimulated with IFN-γ+IL-4 [6]. In our study, the MTHC-D2 cells, that were similar to these IL-12 p70 producing spleen DCs, were also stimulated with IFN-γ+IL-4, were $CD8\alpha^{high}$, and had a similar pattern of p40 gene expression as the splenic DCs. Another correlation is the *ex vivo* DCs that produce the least amount of IL-12 p70 but produce the most amount of p40 homodimer, are either CD8α- splenic DCs [53], or $CD11c^{+}$ splenic DCs stimulated with IFN-γ [6]. In our study, the MTHC-D2 cells that had a similar pattern of p40 expression as the splenic DCs, were also stimulated with IFN-γ, and were $CD8\alpha^{low}$. The expression of DEC-205 by MTHC-D2 cells also correlates with the splenic DCs, where this molecule was marginally greater in the IFN-γ+IL-4+TNF-α stimulated $CD8\alpha^{high}$ MTHC-D2 cells compared with the IFN-γ+TNF-α $CD8\alpha^{low}$ MTHC-D2 cells. This is similar to the *ex vivo* CD8α+ DCs that also express higher levels of this molecule compared with the CD8α- DCs [54]. A correlation between *ex vivo* spleen DC subtype, production of IFN-γ, and MTHC-D2 response to IFN-γ is also evident. The splenic DC subtype, which produces large amounts of IFN-γ are CD8α-CD4- [53], whereas the MTHC-D2 cells that are $CD8\alpha^{low}CD4^{-}$ are stimulated with IFN-γ. The data with MTHC-D2 suggest that IFN-γ causes CD8α expression to be low, and therefore this cytokine may contribute to the CD8α- DC phenotype *in vivo*. Despite these correlations there are also differences in their cell surface phenotype, including the IFN-γ+IL-4+TNF-α stimulated MTHC-D2 cells that are $CD8\alpha^{high}$ express similar levels of CD11b to the IFN-γ+TNF-α MTHC-D2 cells that are $CD8\alpha^{low}$. Whereas, the CD8α+ *ex vivo* DCs express lower levels of CD11b compared to the CD8α- DCs [54]. It cannot be ruled out that the differences in expression of CD11b may be under the control of other cytokines or immunostimulatory factors not used in the MTHC-D2 study.

Other recent studies of the CD8α+ and CD8α- splenic DC subtypes have began to shed light on the different functions of these cells. There is an association of the CD8α+ subtype with cross-presenting antigen on MHC I molecules to CD8 T cells, and the CD8α- subtype with MHC II-restricted direct antigen presentation to CD4 T cells [55] [56]. Interestingly, another correlation exists between cytokine stimulation, and expression of CD8α, MHC I/II in the MTHC-D2 cells that supports the findings of the *ex vivo* DCs (Table 3). The IL-4+TNF-α stimulated MTHC-D2 cells were $CD8\alpha^{high}$ and expressed higher levels of MHC I than the $CD8\alpha^{low}$ IFN-γ+TNF-α stimulated MTHC-D2 cells by northern

analysis. Also, cDNA subtraction and RT-PCR found higher levels of MHC II gene expression in the CD8α ^{low} IFN-γ+TNF-α stimulated MTHC-D2 cells compared with the CD8α ^{high} IFN-γ+IL-4+TNF-α and IL-4+TNF-α stimulated MTHC-D2 cells. Regulation of MHC I and MHC II expression in response to these cytokines may enable DC subtypes to process and present antigen differently to T cells. It is possible the greater phagocytic ability, found in association with the IFN-γ+IL-4+TNF-α stimulated CD8α ^{high} MTHC-D2 cells, could potentially facilitate a cross-presenting function enabling the cells to more efficiently take up antigen contained within cells undergoing apoptosis. Preliminary studies within our laboratory support this hypothesis where stimulation of a DC cell line, JAWS II (ATCC CRL-11904), with IFN-γ+IL-4+TNF-α enhanced their capacity to cross-present SINFEKEL antigen from JAWS II cells infected with a recombinant vaccinia expressing this antigen, compared with IFN-γ+TNF-α stimulation (data not shown).

A question that does arise when assessing the effects of IFN-γ and IL-4 on the development of DC associated immunoregulatory properties, is under what circumstances during an immune response would the microenvironment have different representation of these cytokines. It is easy to visualise that under inflammatory conditions induced by a highly virulent pathogen, an abundance of IFN-γ may support maturation of a DC with immunoregulatory properties that is more likely to support CMI. It is also possible during HI or in response to a pathogen that stimulates IL-4 production from DCs [2], that the cytokine microenvironment would support maturation of a DC more likely to support HI development. A microenvironment where both IFN-γ and IL-4 may be found in abundance could occur either in response to a pathogen that induces expression of these cytokines, or during a progressive change between CMI and HI. A number of studies have investigated factors associated with a change from CMI to HI. In a kinetic study of DC IL-12 production, the DCs were found to support Th1 cell proliferation as IL-12 production peaked and subsequently support Th2 development as IL-12 declined [3]. Also, kinetic studies of migrating T helper cells have shown that Th1 IFN-γ producing cells that have migrated to the site of infection, secrete chemoattractants that later recruit Th2 IL-4 producing cells [57]. This cascade of DC and T cell interactions could create a microenvironment containing both IFN-γ and IL-4 that would direct maturation of DCs recruited to the site, and also as our study has indicated, possibly transdifferentiate the IFN-γ stimulated DCs at the site of infection by the sequential addition of IL-4.

The possibility that DCs may transdifferentiate, and in doing so alter their immunoregulatory properties has been suggested [58] but so far has received little attention. More recent studies however, are indicating that DCs have a high degree of functional plasticity through analysis of *ex vivo* CD8α+ and CD8α- DC subtypes, where their potential

to drive Th1 and Th2 responses is thought not to be fixed for each subtype [59]. Another recent study has discovered that polymorphonuclear neutrophils, considered to be end-differentiated, were found *in vivo* and *in vitro* to transdifferentiate, acquiring functional and morphological characteristics of DCs during pathological events or when stimulated with IFN-γ and GM-CSF [60]. This suggests that mature innate immune cells, such as neutraphils and DCs, are capable of changing their functional properties in the presence of pathological and cytokine stimulus. In this study the MTHC-D2 cell line retained its sensitivity to cytokine stimulation. The addition of IFN-γ to IL-4+TNF-α stimulated cells or IL-4 to IFN-γ+TNF-α stimulated cells resulted in a morphological and cell surface marker change (data not shown) to a cell type with the characteristics generated by stimulation with both IFN-γ+IL-4+TNF-α. This occurred much more rapidly (within 6 h) than was required when these cytokines were added together to un-stimulated GM-CSF cultured MTHC-D2 cells (24-48 h). The ability of these cells to change their immunoregulatory properties so rapidly may represent an immune modulating role of DCs that enables these cells to facilitate progression from one state of immunity to another.

It is interesting to speculate that a transdifferentiation mechanism, at the site of infection, may enable DCs to aid progression from CMI to HI as the IFN-γ producing Th1 cells secrete chemokines that attract IL-4 producing Th2 cells [57]. In support of this hypothesis *in vivo* studies have identified a unique DC type that can only be found at the site of inflammation and not in the lymph or spleen tissue [18]. This DC type is described as large, macrophage-like, often containing apoptotic bodies within intracellular vacuoles, is phagocytic, and cross-presents antigen. It is interesting that combining IFN-γ and IL-4 stimulation induced the MTHC-D2 cells to develop similar properties to that described for this cross-presenting DC. It is possible that a DC exposed to both IFN-γ and IL-4 may develop properties that provide an alternative pathway for antigen processing and presentation to adaptive T cells. Particularly where a pathogen induces cytopathic effect or apoptosis of the DCs at the site of infection, such as viral vaccine agents that cause apoptosis of DCs [61].

Unlike the hypothesis presented here, others speculate a role for the IFN-γ plus IL-4 stimulated cross-presenting CD8α+ spleen DC in switching an immune response from HI to CMI [6]. Our studies indicate that transdifferentiation to the IFN-γ plus IL-4 phenotype can be induced by either IFN-γ or IL-4 addition to IL-4+TNF-α or IFN-γ+TNF-α stimulated MTHC-D2 cells. Therefore, this mechanism may aid progression from CMI to HI or from HI to CMI. Others have also speculated that in an environment with high levels of IFN-γ, in the absence of IL-4, production of the IL-12 p40 homodimer from CD8α- DCs may prevent an exaggerated Th1 response from occurring. However, considering that once IL-12 peaks in DCs these cells then support Th2 cell development [3] suggests this type of mechanism may

not be required. In addition, it has now been shown that DCs are still able to induce CMI in the absence of IL-12 p70 [8]. Hence it is possible the IFN-γ stimulated DC only develops during circumstances of an early immune response to a pathogen that stimulates IFN-γ production from innate immune cells, and this DC stimulates CMI using as yet unidentified mechanisms.

In conclusion, the studies presented here suggest in an IFN-γ abundant microenvironment, this cytokine stimulates the development of immunoregulatory properties associated with mature DCs, potent T cell stimulatory capacity, anti-viral mechanisms, and features supportive of CMI. It is interesting to speculate that in the absence of inflammatory stimuli, and in the presence of IL-4, a DC may develop properties common to a phagocyte with little IL-12 production, lower T cell stimulatory capacity, and enhanced ability to take up extracellular pathogens, that together support HI. Finally, at a stage of immunity where Th1 and Th2 cells come together, an environment may exist that enables DCs to enhance phagocytosis for cross-presenting antigen and for antibody mediated endocytic clearance of CMI lysed cells. It is now important to extend these studies by further exploring the immunoregulatory properties induced by IFN-γ+IL-4, and to cross-compare the findings reported here with the response of *ex vivo* DCs to these cytokine combinations. In addition, tracing the immunoregulatory phenotype of DCs at the site of infection throughout the course of an immune response will help to clarify the ongoing role of these cells and how they may support a changing immune response.

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Figure Legends

Figure 1: Morphological analysis of cytokine stimulated MTHC-D2 cells by TEM. MTHC-D2 cells were stimulated for 48 h with effector cytokine combinations and CD40 Ab then processed for TEM analysis. Each cell is shown at 5K magnification. Arrows shown in B indicate release of exosomes, and in D indicate vacuoles and apoptotic bodies inside a vacuole.

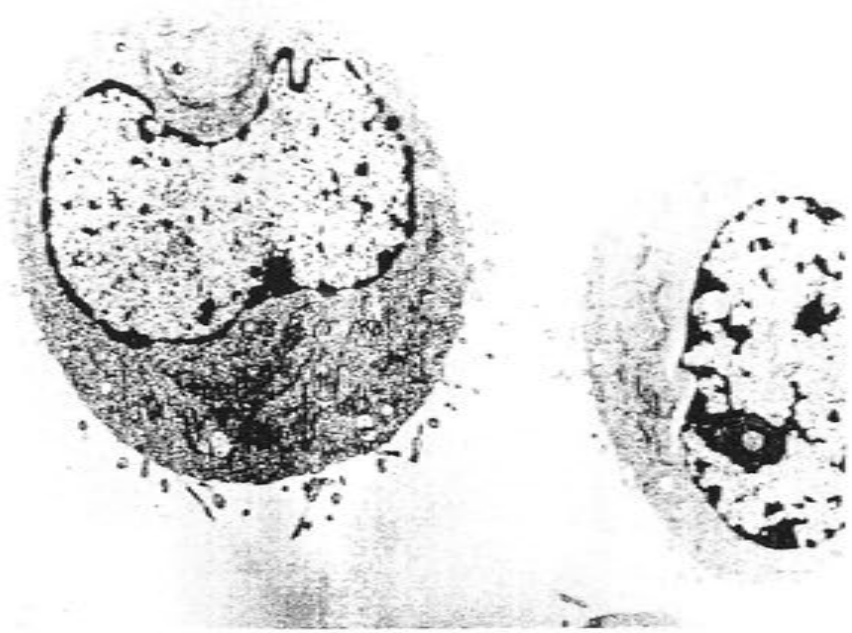
Figure 2: Ability of cytokine stimulated MTHC-D2 cells to phagocytose SRBC's. MTHC-D2 cells were stimulated with cytokines for 48 h, and where indicated, were activated with CD40 Ab for the final 16 h. MTHC-D2 cells were then co-cultured with SRBC's for 1 h then stained and analysed by light microscopy.

Figure 3: Analysis of MTHC-D2 phagocytosis of SRBC's by TEM. MTHC-D2 cells were stimulated with effector cytokine combinations for 48 h followed by co-culture with SRBC's for 1 h and processing for TEM analysis. Each cell type is shown at 5K magnification. Those cells shown were 3a A: un-stimulated MTHC-D2 cells; B: IFN-γ+TNF-α stimulated; C: IL-4+TNF-α stimulated; D: IFN-γ+IL-4+TNF-α stimulated. 3b: IFN-γ+IL-4+TNF-α stimulated cells degrading SRBCs. Arrow heads refer to dendritic processes on the cell surface, whereas arrows refer to SRBCs.

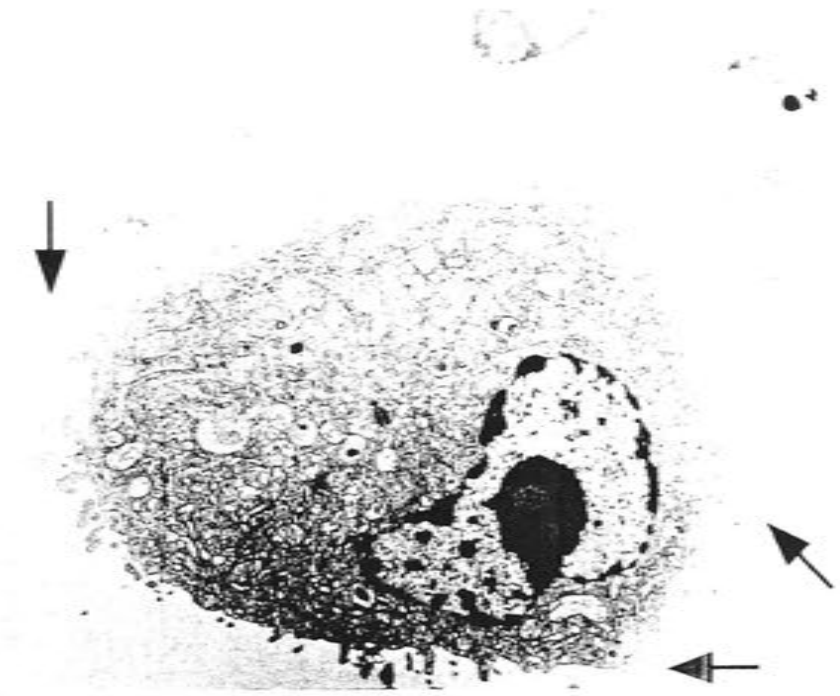
Figure 4. The ability of cytokine stimulated MTHC-D2 cells to induce T cell proliferation. Mixed lymphocyte reactions measured by FACS staining of proliferating T cells were performed using CD90 to detect all proliferating T cells in **MLR 1**, CD8α to detect CD8 T cell proliferation in **MLR 2**, and CD4 to detect CD4 T cell proliferation in **MLR 3**. Un-activated MTHC-D2 cells were used in **MLR 1**, and CD40 Ab activated MTHC-D2 cells were used in **MLR's 2 and 3**. Legend **A** refers to MLR's using un-stimulated MTHC-D2, **B** refers to IFN-γ + TNF-α stimulated MTHC-D2, **C** refers to IL-4 + TNF-α stimulated MTHC-D2 cells, and **D** refers to IFN-γ +IL-4+ TNF-α stimulated MTHC-D2 cells, the negative control refers to MLR reactions containing only T cells.

Figure 5: Differential expression of immunoregulatory genes in response to IFN-γ and IL-4 cytokine stimuli of MTHC-D2. These cells were stimulated with cytokine combinations and CD40 Ab followed by mRNA extraction, first strand cDNA preparation, and semi-quantitative RT-PCR analysis. The genes targeted for expression analysis are listed on the right. Cytokine and activation stimuli of cells representing 1-8 are as follows. (1) GM-CSF, (2) GM-CSF+CD40 Ab, (3) IFN-γ+TNF-α+GM-CSF, (4) IFN-γ+TNF-α+GM-CSF+CD40 Ab, (5) IL-4+TNF-α+GM-CSF, (6) IL-4+TNF-α+GM-CSF+CD40 Ab, (7) IFN-γ+IL-4+TNF-α+GM-CSF, (8) IFN-γ+IL-4+TNF-α+GM-CSF+CD40 Ab.

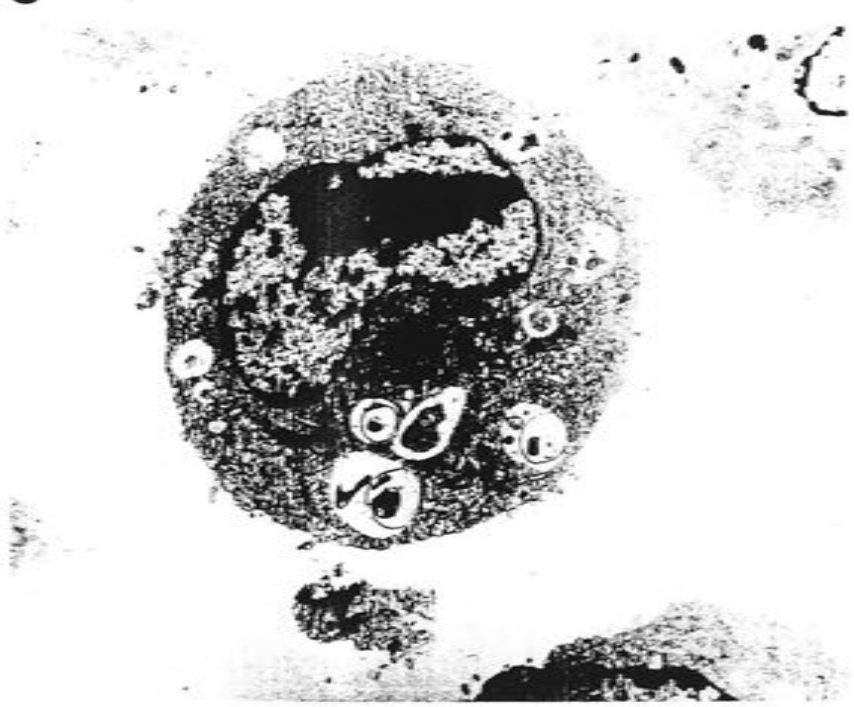
A



B



C



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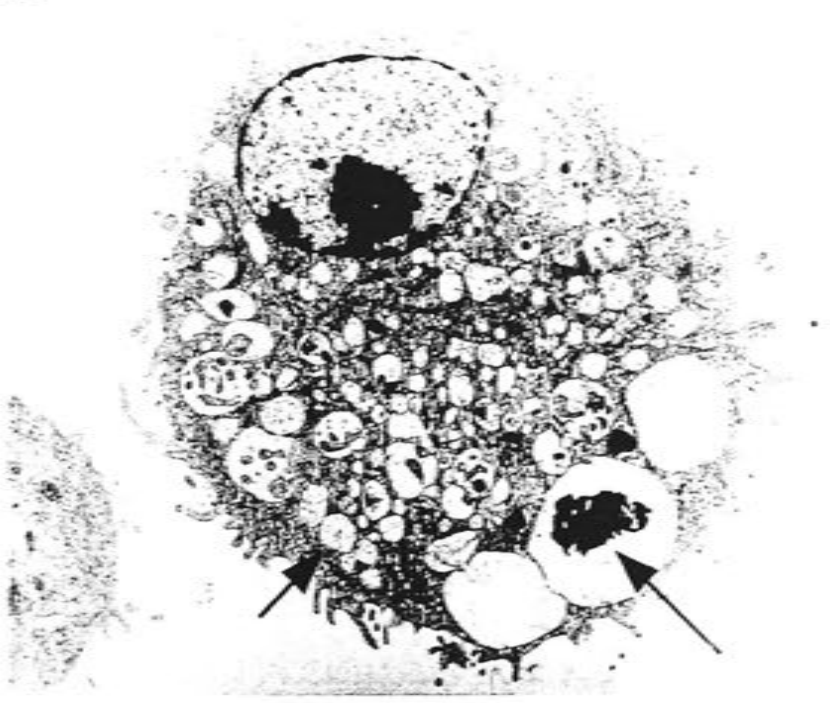


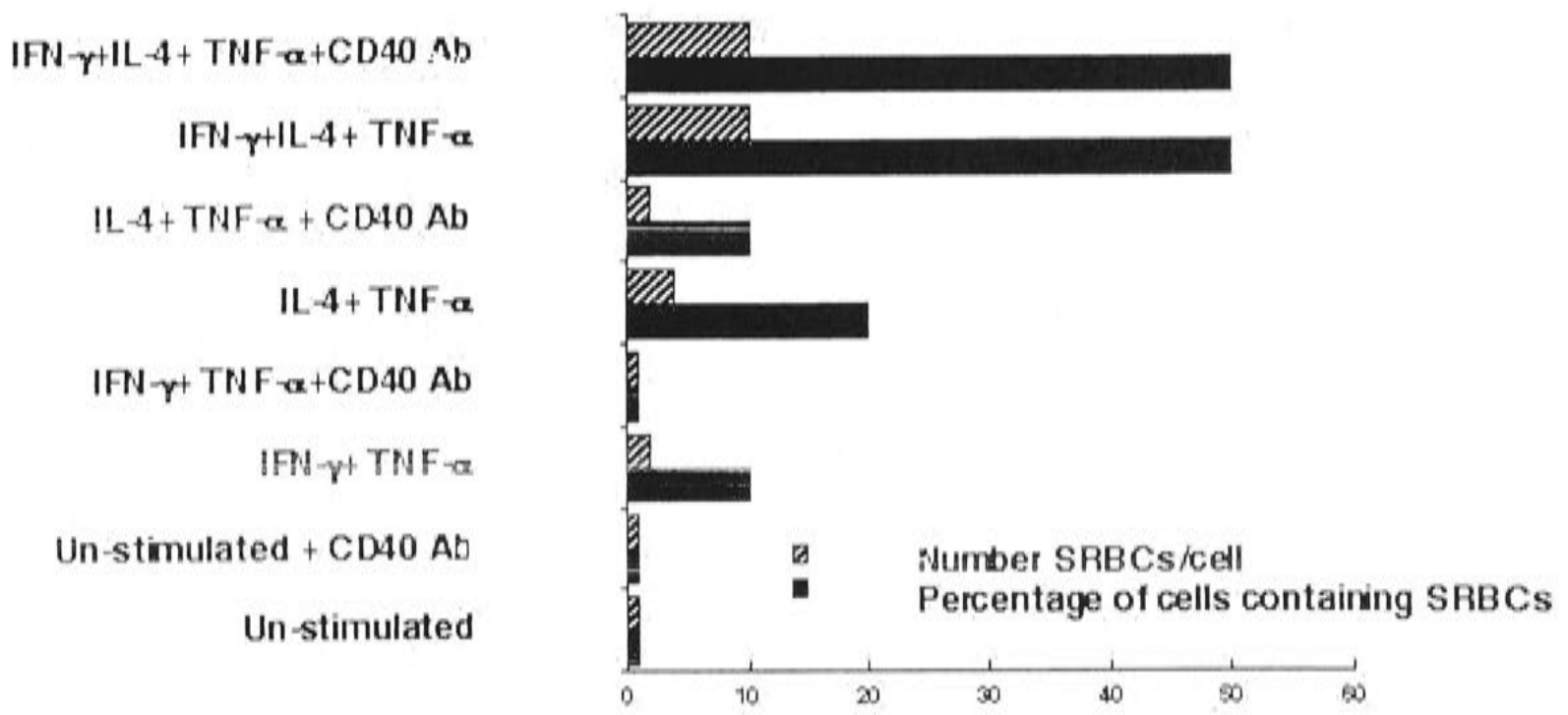
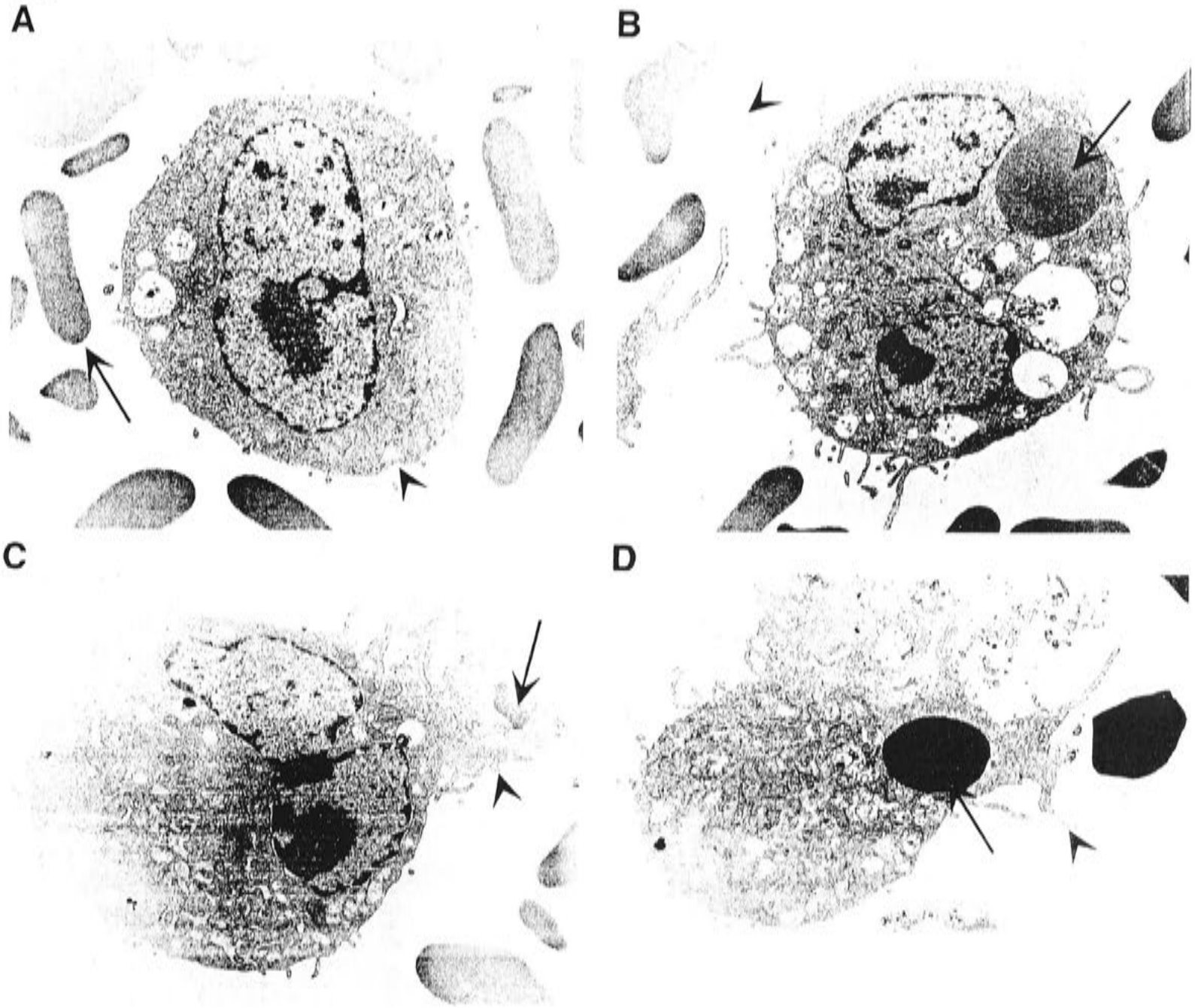
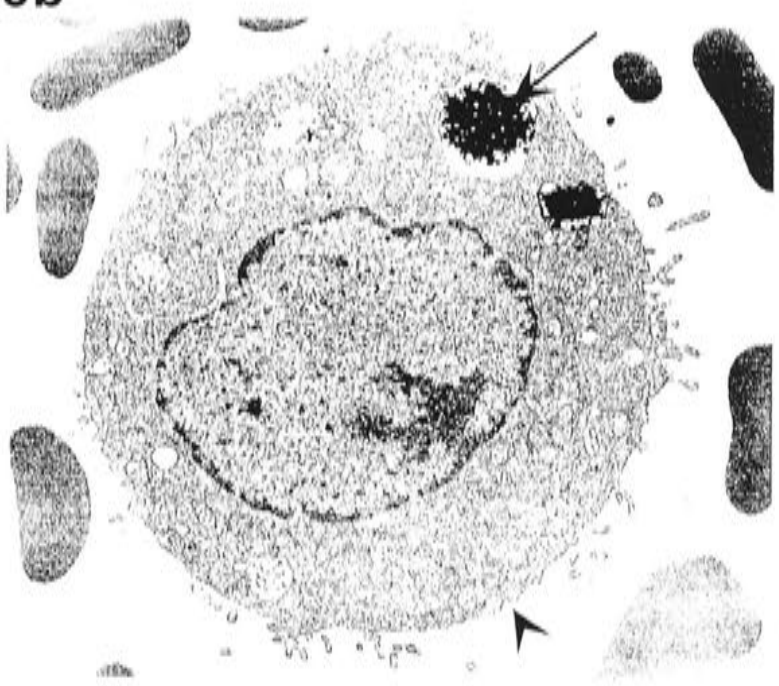

IFN-γ+IL-4+ TNF-α+CD40 Ab
IFN-γ+IL-4+ TNF-α
IL-4+ TNF-α + CD40 Ab
IL-4+ TNF-α
IFN-γ+ TNF-α+CD40 Ab
IFN-γ+ TNF-α
Un-stimulated + CD40 Ab
Un-stimulated
Number SRBCs/cell
Percentage of cells containing SRBCs
0
10
20
30
40
50
60

Figure 2

3a



3b



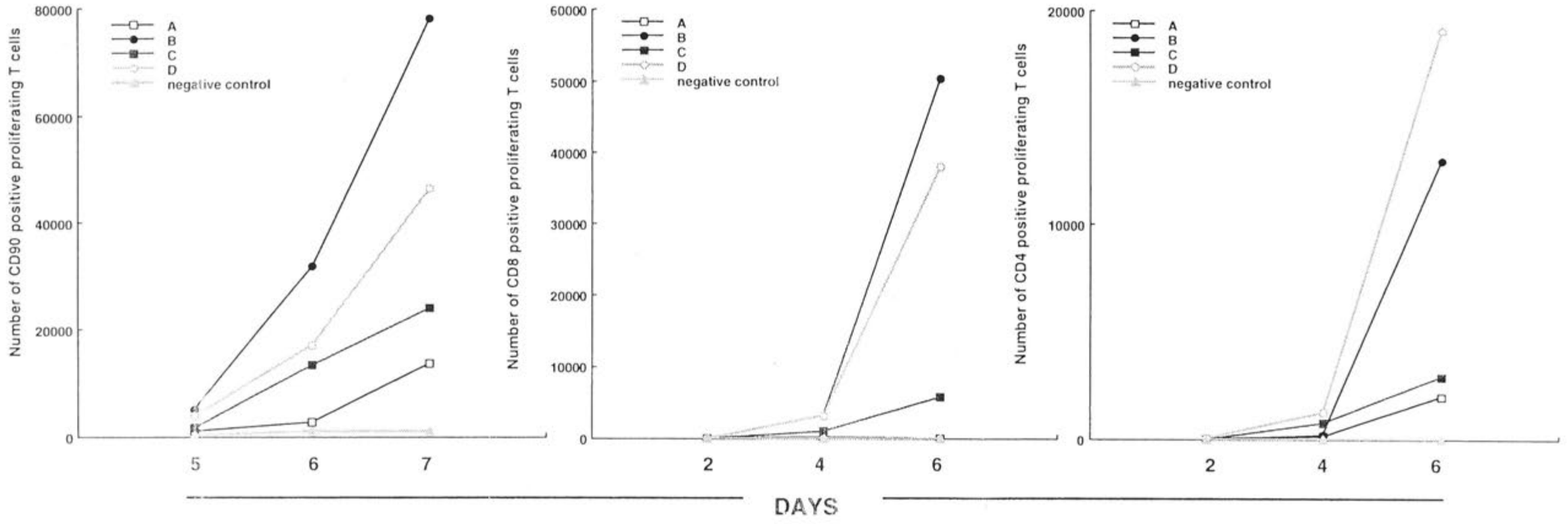

MLR 1
Un-activated MTHC-D2/T cell
Number of CD90 positive proliferating T cells
80000
60000
40000
20000
0
A
B
C
D
negative control
5
6
7
MLR 2
CD40 activated MTHC-D2/CD8 T cell
Number of CD8 positive proliferating T cells
60000
50000
40000
30000
20000
10000
0
A
B
C
D
negative control
2
4
6
MLR 3
CD40 activated MTHC-D2/CD4 T cell
Number of CD4 positive proliferating T cells
20000
10000
0
A
B
C
D
negative control
2
4
6
DAYS

Figure 4.

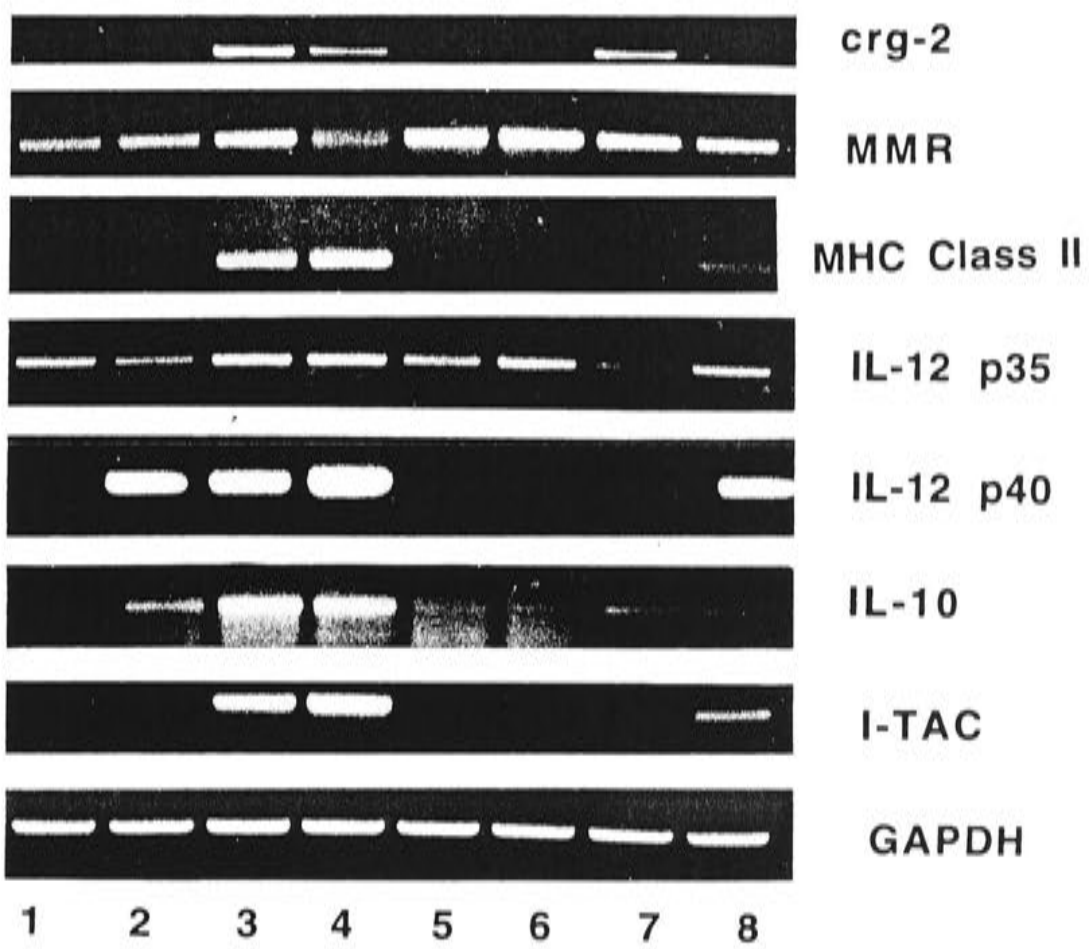

crg-2
MMR
MHC Class II
IL-12 p35
IL-12 p40
IL-10
I-TAC
GAPDH
1
2
3
4
5
6
7
8

Table 1. FACS analysis of cell surface marker expression in response to cytokine and activation stimuli.

Group	Antibody	Geo-Mean fluorescent intensity and SD of cytokine-stimulated MTHC-D2 cells													
		A		A^{CD40}		B		B^{CD40}		C		C^{CD40}		D		D^{CD40}	
Control	No stain	**2**	(0.15)	**2**	(0.07)	**3**	(0.06)	**3**	(0.12)	**3**	(0.11)	**3**	(0.06)	**4**	(0.14)	**3**	(0.14)
Co-stimulatory/ Activation	CD40	**5**	(0.29)	**6**	(0.48)	**15**	(0.53)	**25**	(0.98)	**17**	(2.64)	**14**	(1.04)	**26**	(2.42)	**36**	(1.27)
	CD80	**7**	(0.66)	**8**	(0.58)	**44**	(1.58)	**66**	(1.68)	**29**	(0.60)	**19**	(0.40)	**43**	(2.07)	**89**	(1.83)
	CD86	**3**	(0.05)	**3**	(0.06)	**10**	(0.35)	**18**	(0.28)	**4**	(0.23)	**6**	(0.94)	**7**	(0.51)	**13**	(0.30)
	CD69	**13**	(0.89)	**14**	(0.21)	**60**	(2.04)	**123**	(1.07)	**43**	(0.52)	**42**	(2.42)	**58**	(0.47)	**99**	(2.69)
DC and macrophage markers	CD8α	**7**	(0.43)	**7**	(0.14)	**11**	(0.50)	**13**	(0.81)	**39**	(2.70)	**29**	(2.09)	**35**	(2.10)	**30**	(1.52)
	CD11c	**2**	(0.07)	**2**	(0.13)	**4**	(0.02)	**5**	(0.22)	**10**	(0.68)	**6**	(0.13)	**8**	(0.04)	**18**	(0.13)
	CD11b	**7**	(0.51)	**8**	(0.27)	**17**	(0.35)	**19**	(0.00)	**16**	(0.00)	**13**	(0.00)	**21**	(0.00)	**22**	(0.45)
	DEC-205	**3**	(0.25)	**3**	(0.00)	**7**	(0.14)	**8**	(0.16)	**8**	(0.51)	**9**	(0.00)	**10**	(0.38)	**14**	(0.29)
	CD44	**202**	(5.13)	**225**	(5.72)	**274**	(0.00)	**322**	(8.19)	**340**	(0.01)	**340**	(0.00)	**407**	(0.00)	**505**	(0.00)

MTHC-D2 cells were stimulated with the following cytokine combinations for 48 h and activated with CD40 Ab for the final 16 h followed by antibody staining and FACS analysis. A GM-CSF, B GM-CSF+TNF-α+IFN-γ , C GM-CSF+TNF-α+IL-4 D GM-CSF+TNF-α+IFN-γ+IL-4. SD - Standard Deviation shown in brackets.

TABLE 2. Frequency and Northern analysis of selected known genes up-regulated in cDNA subtracted libraries from cytokine-stimulated MTHC-D2 cells.

Accession/ Medline	cDNA	Gene Category	Subtracted library				Expression in MTHC-D2 cells		
			1	2	3	4	un-stimulated	IFN-γ/ TNF-α	IL-4/ TNF-α
cDNA clones isolated from IFN-γ+TNF-α specific libraries			Clone Frequency						
		Chemokines							
M86829/90368683	Crg-2	IFN-γ inducible chemokine		65	69				
S37648/92289805	RANTES	monocyte chemoattractant		2	4				
M34815/90319087	MIG	monkine induced by IFN-γ		50	28				
NM019494/20302783	I-TAC	IFN-γ induced. T cell chemoattractant		1	3		0	10	0
		IFN-inducible							
NM011940/99425269	Ifi202b	IFN activatable protein		3	3				
AF022371/98028407	Ifi203	IFN-activatable protein		2	5				
NM008329/90008876	Ifi 204	IFN-activatable protein		8	5				
NM008390/ 88311092	IFN regulatory factor 1	Induces IFN genes with anti-viral activity		8	4				
NM016850	IFN regulatory factor 7	Induces IFN-α+β in virally infected cells, and RANTES		8	6				
S77713/95203869	Ifi54	IFN-alpha regulated gene		8	5				
S77715/ 95203869	Ifi56	Viral infection or IFN-γ induced		2	12				
S62227/93274206	LPS-inducible gene	New member of an IFN-inducible gene cluster in macrophages		5	4				
M21117/89096892	Mx1	IFN-induced nuclear protein that inhibits influenza virus replication		7	8				
U15635/95190404	Mg11	IFN-γ induced. Contains GTP binding motif		5	8				
M63961/92091752	Mag-1	IFN-γ inducible protein. Accompanies Th1 cytokine expression.		22	15				
NM013673/97422606	Sp100	IFN-inducible transcription factor. Role in early anti-viral response.		5	1				
		Immune response							
M57890/91035430	Factor B	Alternative complement pathway		8	5				
NM009982/97276897	Cathepsin C	Activation of granzymes and CD8 T cell cytolytic activity		2					
L38281/95237894	Irg-1	Immune responsive gene		9	21				
AJ007971/99077537	IIGP protein	GTPase, infection induced		1			1	10	1
M16238/87175527	Fibrinogen-like protein	In cytotoxic T lymphocytes but not helper T or B lymphocytes.		3	2				
BC004059	Pre-B cell colony-enhancing factor	cytokine acts on early B-lineage precursor cells			1		3	10	7
		Cell surface proteins							
L23638/93314711	CD69	Early activation antigen.		3	1		2	10	3
NM010387/92018224	MHC II	Antigen presenting Major Histocompatability Complex Class II			7				
M63286/91244839	Mannose-6-phosphate receptor	Sorts enzymes to lysosomes and association with phagocytosis		1			5	10	9
		Signalling							
	GTP binding proteins (13 types)	IFN-inducible GTP binding proteins		52	45				
	GBP binding protein (12 types)	IFN-γ regulated genes in macrophage activation		83	64				
NM010260/99074031	GBP-2	Example of GBP protein		4	3		0	10	0
BC010229	Similar to GBP-3	Example of GBP protein		2			10	10	1
AF349678	STAT-1	Signal transducer		10	12		2	10	3
BC004808	Similar to STAT-1	Signal transducer			4		5	10	5
NM019963	STAT-2	Signal transducer			3		2	10	3
		Others							
AF020772/980341601	Importin alpha Q2	Nuclear Transport			1		9	10	6
NM011909/99182491	Ubiquitin specific protease, Usp18	Protein degradation			4		3	10	3
AF068835/99323969	M1204	Oligoadenylate synthetase induced in maturation of dendritic cells			2				

TABLE 2 cont. Frequency and Northern analysis of selected known genes up-regulated in cDNA subtracted libraries from cytokine stimulated MTHC-D2 cells.

Accession/ Medline	cDNA	Gene Category	Subtracted library				Expression in MTHC-D2 cells		
			1	2	3	4	un-stimulated	IFN-γ/ TNF-α	IL-4/ TNF-α
cDNA clones isolated from IL-4+TNF-α specific libraries			**Clone Frequency**						
		Cytokines and Chemokines							
M13177/86168129	TGF-β	Transforming growth factor. Mitogenic factor	2				10	7	9
X06086/88076849	MEP Major excreted protein	A remodelling of extracellular matrix secreted cathepsin	1				6	6	10
L11237/94030619	C10	Related to macrophage inflammatory protein	1			2			
X12531/88258380	MIP	Macrophage inflammatory protein	1			2	8	10	10
S71251/94271193	Chemotactic protein-3	Monocytic chemotatic protein from macrophages	1				3	9	10
		Cell surface proteins							
NM010796/92268032	Mg1 receptor	On surface of macrophages involved in binding to tumor cells	6			6	8	3	10
NM010188/87042761	FcγR	Binds Fc portion of IgG. Mediates endocytosis Ab/Ag complex.	8			8	10	6	9
AF272948/21172992	G-protein-coupled Receptor 84	Chemokine receptor and enhances endocytosis				3	0	10	6
D87967/97223399	SHPS1	Cellular adherence and outgrowth				1			
X66532/92347174	L-14	Lectin involved in cell differentiation	1				2	3	10
NM008625/93043353	Macrophage mannose receptor	Phagocytic receptor for micro-organisms. IFN-γ down regulated				1			
X13335/91197896	MS2	macrophage-specific cysteine-rich transmembrane glycoprotein	1			1	8	7	10
X16834/90063462	Mac-2/L-34 galactoside binding lectin	galactose-specific lectin that binds IgE				2	9	7	10
NM011027	Purinergic receptor, P2X	ligand-gated ion channel				4	7	10	10
S36676/92268032	galactose/N-acetylgalactosamine lectin	Expressed on tumoricidal macrophages	3			4			
		Cytoskeleton							
AC091473/90361737	Actin binding protein	Actin cross-linking phosphoprotein of peripheral cytoplasm	2						
Z31399/95041331	Ccth	Cytosolic protein involved in folding actin and tubulin	2			1			
X60671/93055012	Ezrin	Associates short actin filaments with the plasma membrane	2			2			
NM010354/89327303	Gelsolin gene	Restructures actin filaments inducing cell shape change	2						
U16740/97470757	Capping protein alpha 1 subunit	Actin assembly and cell motility	1						
NM011655/87057644	Beta tubulin	Cytoskeleton re-structuring	1						
NM009609/89127235	Cytoplasmic gamma actin	Supports morphological changes, dendrities and cell motility				3			
AF301152/21448996	CAS	Associates with microtubules in proliferating cells	1				10	5	9
		Lysosomal							
U49351	Lysosomal alpha-glucosidase	carbohydrate-hydrolases				2			
M28541/89384641	Beta-glucoronidase	Lysosomal hydrolase				4			
NM007798/91190267	Cathepsin B	Lysosomal thiol proteinase. Involved in processing of antigen				1			
NM009984/86271744	Cathepsin-L	MHC II antigen processing	1						
NM007800/97362044	Cathepsin-G	MHC II antigen processing				1			
NM010686/96299782	LAPTm5	Lysosomal-associated multitransmembrane protein				2			
M32017/90307738	Lgp-B	Lysosomal membrane glycoprotein				1			
M21050/88320416	Lysozyme M	Expressed strongly in macrophages				1			
		Cell migration							
AF026124/99030428	SAM9	Involved in post-migration of neurons				1			
X85991/95267431	Semaphorin B	Acts as growth cone guidance signals, axonal pathfinding				1			
		Immune response							
K02782/85038854	Complement C3	Binds Ag/Ab complexes and tagets to phagocytes for clearance				3			
X12905/88318954	Complement properdin	Alternative pathway. Enhances complement mediated clearance				1			
		Signalling							
X99644/97133299	TIF1	Reacts with nuclear receptors and transcription factors	4						
X17400/90016295	TIS7	Autocrine factor that amplifies initial ligand induced signal	4						
NM009811/97190206	Caspase-6	Nuclear events of apoptosis	3						
U36277/96235048	I-Kappa B alpha chain	Inhibitor of transcription factor NF-κB				10			
AF232716/20307889	Protein arginine N-methyltransferase 1	Signal transduction	3				10	6	9
NM021420/97304522	serine/threonine kinase 4, Stk4	Signal transduction	1				8	7	10
NM019744	Nuclear receptor co-activator 4	Transcription regulation	1				9	7	10
		Others							
NM011456/97326124	serine protease inhibitor 14	Regulators of extracellular proteolysis	1				5	2	10
NM033075/97124840	G7e	Resembles a viral envelope gene in MHC class III gene region				1	2	10	7
cDNA clones isolated from both the IFN-γ+TNF-α and IL-4+TNF-α specific libraries									
		Cell surface proteins							
D89572/97420681	Ryudocan core protein	Cell adhesian, neurite growth promotion, anticoagulation			2	4	7	9	10
M83312/92105763	CD40	Co-stimulatory molecule			5	3	2	10	3
X52264/89345188	ICAM-1	Intercellular adhesian molecule			2	2	2	10	3
M18466/88088825	Ly-6C.2	Lymphocyte differentiation antigen	1		3	1	7	10	9
		Antigen processing							
U47327/99086740	MHC I	Antigen presenting major histocompatabitility complex class I			7	2	5	7	10
NM009735/84028577	B_2M	Complexes with MHC molecules on the cell surface	1		4	3			
NM011530/91102550	TAP	Transporter gene	3		15	1			
		Cytoskeleton							
M13446/87064538	Alpha-tubulin isotype M-alpha-2	Microtubule formation			1	5	3	10	9
		Phagocytosis							
NM007807/96202527	gp91phox	Transfers electrons from NADPH in respiratory burst oxidase	2		1	2			

Selected cDNA subtracted clones from cytokine-stimulated MTHC-D2 cells having >95% over >100 bp homology with known genes associated with cytokine stimulation, dendritic and macrophage maturation/function, and the immune system are shown in this table. cDNA's are clustered according to which subtracted libraries they were isolated from. Subtracted library 1 : IFN-γ+TNF-α subtracted from IL-4+TNF-α stimulated MTHC-D2 cells; 2 : IL-4+TNF-α subtracted from IFN-γ+TNF-α stimulated MTHC-D2 cells; 3 : Un-stimulated MTHC-D2 cells subtracted from IFN-γ+TNF-α stimulated cells; 4 : Un-stimulated MTHC-D2 cells subtracted from IL-4+TNF-α stimulated cells. The frequency of each clone from the respective libraries is given. Selected cDNA clones were further analysed by northern blot screening to establish the comparative expression levels in each cytokine-stimulated MTHC-D2 cell type. For northern analysis the MTHC-D2 cells were stimulated with cytokines for 6 h followed by mRNA extraction and northern blotting. cDNA clones were radioactively labelled and hybridised to the northerns. PhosphorImager using ImageQuant software was used to quantitate the differential expression. Band intensity values for each northern were normalised to GAPDH and scaled such that the highest band value on all northerns was 10.

Table 3. Similarities between splenic *ex vivo* DCs and cytokine stimulated MTHC-D2 cells.

Cell property	Correlation	
	Ex vivo DCs	MTHC-D2
Similar pattern of p40 gene expression as DCs that produce high levels IL-12 p70	CD8α^{+} IFN-γ+IL-4 stimulated	CD8α ^{high} IFN-γ+IL-4 stimulated
Similar pattern of p40 gene expression as DCs that produce low levels IL-12 p70	CD8α^{-} IFN-γ stimulated	CD8α ^{low} IFN-γ stimulated
Greater levels of DEC-205 expression	CD8α^{+}	CD8α ^{high} IFN-γ+IL-4 stimulated
Expression of CD8α and CD4 in the presence/production of IFN-γ	CD8α^{-} CD4^{-}	CD8α ^{low} CD4^{-}
MHC I expression/function	CD8α^{+} DCs are more efficient than CD8α^{-} DCs at cross-presenting antigen on MHC I to CD8 T cells	CD8α ^{high}, IL-4 stimulated cells express greater levels of MHC I than CD8α^{low} IFN-γ stimulated cells
MHC II expression/function	CD8α^{-} DCs are more efficient than CD8α^{+} DCs at directly presenting antigen on MHC II to CD4 T cells	CD8α ^{low}, IFN-γ stimulated cells express greater levels of MHC II than CD8α^{high} IL-4 stimulated cells